THE PATHOLOGY AND IMMUNOLOGY OF PARATUBERCULOSIS IN SHEEP AND GOATS

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Declaration

I declare that this thesis is my own composition and that the work described is my own except where clearly stated.

David Little

February, 1997

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ABSTRACT OF THESIS

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Mycobacterium avium subspecies *paratuberculosis* (*Map*) infects macrophages and causes severe lesions of chronic granulomatous enteritis in sheep and other ruminants (Johne's disease). In this study, ovine paratuberculosis cases were examined histologically, and the presence of two distinct forms of intestinal pathology confirmed, characterised by either multibacillary lesions which exhibited a positive correlation with the presence of a marked macrophage infiltrate (lepromatous cases), or paucibacillary lesions which showed correlation with a cellular infiltrate which was lymphocytic in nature (tuberculoid cases). The presence of acid-fast bacteria was also found to correlate with evidence of serum antibody. Genomic DNA was extracted from the ileum of infected sheep and polymerase chain reaction (PCR) performed using IS900 primers to confirm *Map* infection.

Immunoperoxidase staining of ileum demonstrated that the lymphocyte populations differed in density and relative percentages between both histological groups. The tuberculoid group had higher densities of CD4+, CD8+ and γδTCR+ subsets, and the lepromatous group lower densities of CD4+ and CD8+ subsets, when compared with control animals. Tuberculoid cases were associated with an increase in the relative percentage of CD4+ lymphocytes, whereas lepromatous cases had an increased relative percentage of γδTCR+ cells.

Flow cytometry of lamina propria lymphocytes (LPL) isolated from the ileum of infected and control animals confirmed increased percentages of γδTCR+ cells in lepromatous cases than in controls, and higher percentages of CD8+ and γδTCR+ cells than in tuberculoid cases, which had correspondingly higher percentages of CD4+ cells. Higher percentages of γδTCR+ cells were also noted in mesenteric lymph node lymphocytes (MLNL) from lepromatous cases compared with control animals. Peripheral blood lymphocytes (PBL) of infected animals had increased percentages of B cells, and an associated increase in the percentage of MHC Class II positive cells compared with normal controls.

The relationship of histological lesions to serum antibody, *Map* antigen-specific lymphocyte proliferation and cytokine production was investigated. LPL, MLNL, and PBL were cultured with *Map* PPD, and proliferative responses measured by incorporation of tritiated thymidine. Interferon (IFN)-γ and interleukin (IL)-2 production were measured by ELISA and bioassay respectively. Humoral responses were measured by serum antibody ELISA. A range of immune responses was seen that corresponded to the type of histopathology present, with animals being divisible into two groups. One group was characterised by dominant cell mediated immunity (CMI), lower humoral responses and higher levels of IFN-γ and IL-2, suggesting a Th1 (CMI help) like response. The other group had low antigen-specific proliferation, low IFN-γ and IL-2 and higher antibody levels suggesting a Th2 (B cell help) like response. These groups corresponded to the recognised tuberculoid and lepromatous types of intestinal histopathology respectively, and suggest different pathogenic mechanisms for each form of the disease.

PBL from infected sheep and goats were subjected to CD4+, CD8+ and γδTCR+ subset depletion using magnetic activated cell sorting (MACS) technique. Proliferative responses to *Map* PPD were diminished in cell cultures which had been CD4+ T-cell depleted.

RNA was extracted from the ileum of infected and control sheep, reverse transcribed to cDNA and subjected to PCR using primers for the amplification and detection of mRNA for the cytokines IFNγ, IL-4, and IL-10 and for IL-2 receptor. mRNA for all cytokines was detectable in both groups of infected animals, however higher levels of IL-10 mRNA were present in lepromatous than tuberculoid tissues.

The findings of this study suggest that ovine paratuberculosis is a disease with an immunological spectrum broadly comparable with that described for leprosy, with an apparent Th1 like response in the tuberculoid form and a Th2 like response in the lepromatous form.

Use this side only

Dedication

To Mum, Dad, and Marwan,
whom I especially thank for their support.

"The higher we climb the hill of knowledge, the steeper and more difficult becomes the ascent."
Attributed to Sir John MacFadyean, 1898, on the subject of Jaagsiekte, another slow-onset infection of sheep.

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Publications arising from this thesis

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Burrells C., Clarke C.J., Colston A., Kay J.M., Little D. and Sharp J.M. A study of immunological responses of sheep clinically affected with paratuberculosis (Johne's disease). The relationship of blood, mesenteric lymph node and intestinal lymphocyte responses to gross and microscopic pathology. (Manuscript in preparation)

Burrells C., Clarke C.J., Colston A., Kay J.M., Little D. and Sharp J.M. Interferon-gamma and interleukin-2 release by lymphocytes derived from the blood, mesenteric lymph nodes and intestines of normal sheep and those affected with paratuberculosis (Johne's disease). (Manuscript in preparation)

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ABBREVIATIONS

AF	Acid fast
AFB	Acid-fast bacilli
AGID	Agar gel immunodiffusion test
AIDS	Acquired immune deficiency syndrome
APC	Antigen presenting cell
BCG	Bacillus Calmette Guérin
bp	Base pairs
BSA	Bovine serum albumin
cDNA	Complementary DNA
CFT	Complement fixation test
CMI	Cell mediated immunity
COD	Corrected optical density
ConA	Concavalin A
cpm	Counts per minute
DAB	Diaminobenzidine
DTH	Delayed type hypersensitivity
EAL	Epithelial associated lymphocyte
EAU	ELISA antibody units
EC	Epithelial cells
ELISA	Enzyme linked immunosorbent assay
ENL	Erythema nodosum leprosum
FACS	Fluorescence-activated cell sorting
FITC	Fluorescein isothiocyanate
FCS	Foetal calf serum
FSC	Forward scatter
GALT	Gut associated lymphoid tissue
HBSS	Hanks's buffered saline solution
HE	Haematoxylin and eosin
HIV	Human immunodeficiency virus
ICLN	Ileocaecal lymph node
IEL	Intraepithelial lymphocytes
IFN	Interferon
IL	Interleukin
kb	Kilobase
LAM	Lipoarabinomannan

LFA-1	Lymphocyte function antigen-1
LN	Lymph node
LP	Lamina propria
LPL	Lamina propria lymphocytes
LSA	Lymphocyte stimulation assay
MAb	Monoclonal antibody
MAC	*Mycobacterium avium* complex
Map	*Mycobacterium avium paratuberculosis*
Mas	*Mycobacterium avium silvaticum*
MB	Multibacillary
MHC	Major histocompatability complex
MLN	Mesenteric lymph node
MLNL	Mesenteric lymph node lymphocyte(s)
mRNA	Messenger RNA
MW	Molecular weight
NK	Natural killer
OD	Optical density
PB	Paucibacillary
PBL	Peripheral blood lymphocyte(s)
PBS	Phosphate buffered saline
PMN	Polymorphonuclear neutrophil
PNK	Polynucleotide kinase
PP	Peyer's patch
PPD	Purified protein derivative
RNase	Ribonuclease
RNI	Reactive nitrogen intermediates
ROI	Reactive oxygen intermediates
RPMI	Rosewell Park Memorial Institute media
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
SI	Stimulation index
SSC	Side scatter
TCR	T-cell receptor
Th	T helper
TNF-	Tumour necrosis factor-
ZN	Ziehl Neelsen stain

CHAPTER ONE

INTRODUCTION

1.1 GENERAL INTRODUCTION

Paratuberculosis, or Johne's disease, is an intestinal mycobacteriosis of ruminants which is manifest as a chronic granulomatous enteritis, and is the result of infection by *Mycobacterium avium* subspecies *paratuberculosis* (*Map*) (Chiodini *et al.*, 1984; Thorel *et al.*, 1990). The historical literature concerning paratuberculosis was recently exhaustively reviewed and catalogued by Chiodini (1993). Johne's disease was so named as a result of the work in 1895 of Johne and Frothingham, who demonstrated the presence of acid-fast bacilli (AFB) in sections of ileum from cattle with enteritis, and decided that it was an atypical form of tuberculosis. The disease was called pseudotuberculosis by Bang in 1906, who re-evaluated the condition and decided that the causative organisms were not in fact tubercle bacilli. M'Fadyean in 1907 coined the name Johne's disease, and in 1912 Twort isolated and cultured a mycobacterium, which was subsequently shown to produce enteritis after experimental inoculation of cattle.

Paratuberculosis is a disease of significance to both cattle and sheep farming industries, with high incidence in many areas of the world, and results in considerable economic loss (reviewed by Hutchinson, 1996). Ovine paratuberculosis may be quite widespread, especially within certain geographical areas. A survey of flocks in North-Eastern Spain detected infected individuals in samples of 31% and 24% of flocks tested, depending on which serological assay was used (Aduriz *et al.,* 1994).

1.2 THE ORGANISM

1.2.1 The genus *Mycobacterium*

The genus *Mycobacterium* is part of the wider CMN (*Corynebacterium*, *Mycobacterium*, *Nocardia*) taxonomic group. The genus includes several pathogenic bacteria: *M. bovis*, *M. avium*, *M. farcinogenes*, *M. lepraemurium*, (primarily animal pathogens) and *M. tuberculosis* and *M. leprae* (human pathogens), as well as a number of non-pathogenic, environmental bacteria and saprophytes. *Mycobacterium avium* subspecies *paratuberculosis* was formerly named *M. paratuberculosis*, but has recently been taxonomically subspecified to *M. avium*, with which it shares greater than 99% DNA homology (Cocito *et al.,* 1994).

1.2.2 *Mycobacterium avium* subspecies *paratuberculosis*

The microbiological characteristics of *Map* have recently been thoroughly reviewed by Cocito *et al.,* (1994). In brief, *Map* is an aerobic Gram-positive, acid-fast (resists the decolourisation effect of acid-alcohol after staining with hot carbol-fuchsin) short, thick rod measuring about 0.5-1.5 μm, and is a facultative intracellular pathogen. The bacilli can be demonstrated intracellularly in clumps in tissues and in faeces. Mycobacteria possess a characteristic cell wall with high lipid content (60%) composed of waxes and glycoproteins, which is responsible for the hydrophobic nature and resistance to acids (hence its acid-fast staining properties), disinfectants, antibodies, and desiccation. Pathogenic mycobacteria are generally dependent on organically complexed iron for growth. Mycobacterial siderophores (iron-carrier molecules) known as exochelins are responsible for removal of organic iron from their mammalian equivalents (transferrin/lactoferrin) and transport across the bacterial cell

wall. The iron is then chelated and stored by their intracellular equivalents, the mycobactins, which, being of lipid composition and hydrophobic, are in association with the cell membrane. Exogenous mycobactin P or J is required for *in vitro* culture of *Map*, and although this mycobactin dependency was initially considered to be pathognomonic for *Map*, other subspecies of the *M. avium* complex (MAC) have since been shown to have the same characteristic (notably *M. a. silvaticum*), and some strains of *Map* have been shown to be non-dependent. Bacterial colonies are small (1-5 mm), firm, smooth and raised with a glistening, white rough-smooth appearance, reaching 1-2 mm by 12-16 weeks with some strains producing yellow pigment. Culture is primarily performed on Herrold's egg yolk medium (HEYM) supplemented with mycobactin, or on Watson-Reid agar plates. *Map* is exceptionally slow growing, even within a genus which is in general slow growing, with culture time being in the order of 4-16 weeks, although more rapid culture has been reported if mycobactin-J is substituted for mycobactin-P. *Map* was determined to be a subspecies of the MAC of organisms on the basis of genomic restriction fragment length polymorphism (RFLP) analysis (Thorel *et al.,* 1990). The discovery of IS900, a 1.45 kb insertion sequence, has provided a useful tool in identification of *Map* , since it has been found to be *Map* specific, with 15-20 copies being present in the bacterial genome (Green *et al.,* 1989; Vary *et al.,* 1990). Its expression product is the p43 protein (Tizard *et al.,* 1992).

1.2.3 Host range

All ruminants, both domestic and wild, are susceptible to paratuberculosis infection, including cattle, sheep, goats, deer, camels, and buffalo (Chiodini *et al.,* 1984; Williams *et al.,* 1983). *Map*-induced disease has been described in a number of monogastric species including horses and swine (Larsen *et al.,* 1971, 1972), and laboratory animals can be infected experimentally. Beige and C57BL mice have been

used as experimental models of paratuberculosis (Stabel *et al.,* 1996; Veazey *et al.,* 1995). Lesions of paratuberculosis have been described in wild rabbits, and the rabbit population may prove to be a significant reservoir of infection for ruminants (Angus, 1990). A recent study revealed that 67% of wild rabbits surveyed in Eastern Scotland were found to have lesions of paratuberculosis, and suggested that this may have implications for both epidemiology and control of the disease in ruminants (Greig *et al.*, 1997). Disease caused by *Map* is not considered to occur in humans, but in recent years a battery of evidence has emerged suggesting that mycobacteria are involved in the pathogenesis of Crohn's disease (reviewed by Thompson, 1994). Mycobacteria, including spheroplast forms, have been observed in, and isolated from the lesions of Crohn's disease. More specifically, there is intense speculation on the involvement of *Map* in Crohn's disease, with many workers producing contradictory results for the culture of *Map*, the presence of *Map*-specific DNA, and *Map*-specific serological responses, and the involvement of *Map* continues to be the subject of debate. Nonetheless, there is some evidence which serves to implicate this organism in the pathogenesis of Crohn's disease, both on a serological basis (Kreuzpaintner *et al.,* 1995) and on the demonstration of IS900 in lesions (Fidler *et al.,* 1994; Sanderson *et al.,* 1992). Van Kruiningen *et al.,* (1986) reproduced disease akin to paratuberculosis in goats by inoculation of a mycobacterium isolated from a human Crohn's disease patient. The presence of *Map* in commercially available cows' milk (Millar *et al.,* 1994), and the failure to inactivate the organisms by pasteurisation (Grant *et al.,* 1996) may add an extra possibility for exposure of susceptible human potential hosts to this pathogen.

1.3 PARATUBERCULOSIS

1.3.1 Transmission and pathogenesis

Transmission of paratuberculosis is thought to occur most frequently by the oral route following ingestion of the organism from faecal contamination of the environment, or on the teats of the dam, while the juvenile animal is still sucking. The portal of entry for the organism appears to be the Peyer's patch of the distal ileum, with the M cells and sub-epithelial macrophages of this area being responsible for the uptake of the organism into the gut (Momotani *et al.,* 1988). The involution of the Peyer's patch as the young ruminant matures may be responsible for the age-related resistance that has been noted in these species (Larsen *et al.,* 1975). Earliest lesions have been found in the ileal Peyer's patches and also in the pharyngeal tonsillar tissues (Gilmour *et al.,* 1965; Payne and Deans Rankin, 1961a, 1961b). Infected bovine foetuses have been described (Sweeney *et al.,* 1992), although a survey of ovine foetuses failed to demonstrate the presence of *Map* (Bastianello *et al.,* 1994). *Map* has also been isolated from the milk of a cow with clinical disease, and this is another potential source of infective organisms for calves (Taylor *et al.,* 1981). Three clinical stages of infection have been proposed (Cocito *et al.,* 1994; Lepper *et al.,* 1989) which are characterised by being subclinical without detectable excretion of organisms in the faeces, subclinical with excretion, and clinical with excretion. The subclinical period is a long one, generally at least 18 months, with clinical cases in cattle generally occurring between 3 and 5 years of age, and rarely in younger animals (Chiodini *et al.,* 1984).

The macrophages of the intestine and draining lymph nodes become the habitat for *Map* through phagocytosis of the organism, and characteristic of intracellular infections is that the bacteria are in a position of privilege from which the host's humoral defences can be evaded (Chiodini, 1991). While succeeding in the evasion of antibody responses, the processing and presentation of mycobacterial antigen by host macrophages allows recognition of infection by T lymphocytes. Chemotactic factors are released by the host macrophage and influx of macrophages and lymphocytes into

the affected tissue is consequential in an attempt to achieve clearance of the pathogen. This often results in a substantial infiltrate of immune cells, either of macrophages or of mainly lymphoid cells, and the presence of the infiltrate may result in distortion of normal intestinal structure and thereby impair gut function. The accumulation of immune cells in the intestinal lamina propria causes thickening of the mucosa with flattening of the villi and a corresponding functional impairment of absorption. It appears that the loss of function and tissue damage are in the main attributable to the host's response to the pathogen, and not due to any directly or intrinsically toxic effect of the mycobacterium itself, such as has been noted in *M. tuberculosis* infection (Rook *et al.,* 1991). Delayed-type hypersensitivity responses raised against mycobacterial antigens are the likely cause of the pathological changes in the host's tissues, and are demonstrable *in vivo* by intradermal testing (Bendixen, 1978). Early stage infections are characterised by a predominantly cell-mediated immune response which becomes increasingly humoral as the disease progresses with eventual anergy being described in individual cases (Chiodini *et al.,* 1984). Impairment of lymphatic drainage with resultant congestion and impaired metabolite uptake results in a net secretion of plasma into the gut lumen, with malabsorption and negative energy and nitrogen balance as consequences (Patterson *et al.,* 1967). There are still considerable gaps in knowledge of the pathogenesis of paratuberculosis, and this is in part due to difficulties in detection of animals in the early and subclinical stages of the disease. Furthermore, the extended period between infection and clinical disease make paratuberculosis less than well suited to experimental infection studies.

1.3.2 Clinical manifestations

Johne's disease is a disease of very slow onset, with animals infected at the neonatal stage progressing to clinical disease in adulthood. Consequently, disease

usually occurs within the 3-5 years age-group. Clinical signs appear terminally, and are the culmination of a long subclinical infection. The primary clinical sign is ill thrift, with initial failure to gain weight and eventual weight loss leading to emaciation. In cattle, poor skin and coat condition may be evident (Chiodini *et al.,* 1984), and wool slip has been reported in sheep (Stamp and Watt, 1954). Periods of intermittent pyrexia have been noted. The major clinical sign of bovine paratuberculosis is chronic, intractable diarrhoea and is again associated with the advanced stages of disease. Diarrhoea is present in few sheep, although faeces may be soft (Carrigan and Seaman, 1990; Stamp and Watt, 1954). Animals are rarely moribund until the terminal stages, when ventral or intermandibular oedema may occur, due to the development of protein-losing enteropathy (Patterson *et al.,* 1967). Significantly lowered serum albumin and total-protein levels have been described in sheep with clinical paratuberculosis, although peripheral oedema did not feature in the animals examined (Scott *et al.,* 1995).

1.3.3 Pathology

The pathological changes in paratuberculosis have been catalogued by a number of authors, for bovine (Buergelt *et al.,* 1978; Chiodini *et al.,* 1984; Barker *et al.,* 1993), ovine (Stamp and Watt, 1954; Rajya and Singh, 1961; Nisbet *et al.,* 1962; Reddy *et al.,* 1984; Carrigan and Seaman, 1990; Pérez *et al.,* 1996) and caprine species (Lenghaus *et al.,* 1977; Fodstad and Gunnarsson, 1979; Morin, 1982; Collins *et al.,* 1984). At necropsy, animals are often found to be emaciated and frequently have evidence of systemic oedema, especially of the mesentery, with significant enlargement of the regional, mesenteric lymph nodes (MLN). The intestinal serosa often appears oedematous with prominent, glassy lymphatic vessels. The distal small intestine, in particular the distal ileum and the region of the ileocaecal valve, is the area

most frequently found to be grossly affected. Although any part of the intestinal tract may be involved, in other areas of the small and large intestines gross lesions are found more sporadically. In affected areas, the intestinal wall appears thickened, with matt velvety and hyperaemic mucosal surface, which may be granular in appearance. Transverse corrugations of the mucosa are frequently described, and these corrugations do not flatten when the gut is stretched. Some strains of bacteria give rise to a yellow pigment which suffuses the mucosa. Lesions can be either localised or diffuse throughout the gut, however in some cases macroscopic changes are absent, despite the presence of histological lesions. Histologically, clumps of acid-fast staining bacilli are seen within macrophages, and intestinal villi are distorted, thickened, and stubby in appearance. There is a diffuse infiltrate of immune cells, including macrophages which have abundant foamy cytoplasm and are frequently packed with AFB. Areas of caseous necrosis and calcification have been noted, especially in sheep and goats, in which an histologically different form of the disease has been described, which is associated with paucity of AFB and a predominantly lymphocytic infiltrate. Granulomatous lesions with intracellular AFB have often been found in mesenteric lymph nodes and occasionally in the liver.

1.3.4 *Ante-mortem* diagnosis

Diagnosis of *Map* infection is based on either the detection of the organism, or demonstration of host response, and methods by which this can be achieved have been the subject of considerable research. The currently available diagnostic techniques have been reviewed by Kreeger, (1991), Cocito *et al.* (1994) and Collins, (1994).

Culture of *Map* from faeces continues to be a frequently employed procedure (reviewed by Whipple *et al.,* 1991). An advantage of this method is its high

specificity. It also has the advantage of detecting animals which are likely to be a source of infection within the group, and facilitates control of infection by removal of faecal excretors. It is a technically straightforward technique, but does require decontamination of the sample to prevent bacterial overgrowth, and this may affect the viability of the organism. The major disadvantage is the time before a result is available, with 12-16 weeks required for culture of *Map* colonies. Culture of *Map* from faeces, needless to say, is also dependent on the presence of organism in the sample, and if animals are not shedding high numbers, or are excreting organisms intermittently, then this will lower the sensitivity of the test, which has been estimated to be approximately 50% (Collins, 1994). Furthermore, culture of *Map* from the faeces of ovine paratuberculosis cases is reportedly more difficult and less sensitive (Juste *et al.,* 1991). In addition to conventional culture, radiometric methods of culture are available (BACTEC system) which are based on the measurement of radiolabelled CO_2 released by growing mycobacteria into the culture broth (Collins, 1994). This method is reported to have higher sensitivity with a lower limit of detection, and to be more rapid than conventional culture, with the time for detection of growth dependent on the numbers of *Map* organisms in the inoculum. At present, however, this technique is not in widespread use. Detection of *Map* in faeces is also possible using *Map*-specific DNA probes. This technique is rapid, and highly specific, but has a lower sensitivity and requires complicated decontamination procedures to prevent interference in the assay by faecal contaminants. Contamination by PCR products is also a potential problem, and this method is relatively costly.

Methods available for the detection of host responses are based on serological evidence of infection, or on the demonstration of cell-mediated immune responses. Serological testing methods have the disadvantage of low sensitivity for the detection of animals in the early stages of the disease, which in general do not show strong antibody responses. Three such serology-based tests are commonly in use. The first

serological test to be evaluated was the complement fixation test (CFT), and continues to be a recognised procedure for detection of paratuberculosis. Some reports have considered this test to have relatively low sensitivity, and low specificity, and is relatively speaking more complicated to perform than newer forms of serological testing. The agar gel immunodiffusion (AGID) test reportedly has a higher threshold of antibody detection than CFT or ELISA (enzyme linked immunosorbent assay) which results in a lower sensitivity for this method, although it has higher specificity. As with other serological tests, many animals in subclinical stages of infection may go undetected. The AGID test has been frequently used for detection of paratuberculosis in sheep and goats (Clarke *et al.,* 1996; Shulaw *et al.*, 1993; Sherman and Gezon, 1980). The AGID test is simple, and inexpensive to perform and no species-specific reagents are required. Results are available in 24-48 hours, but can only be classified as positive or negative, with no quantitation of results possible. The absorbed ELISA test uses the principle of pre-absorption of non-specific antibody by incubation with *Mycobacterium phlei,* thereby increasing the specificity of this test. Detection of *Map*-specific antibody is rapid, and the result can be quantitative as well as simply positive or negative. The optical density of the sample correlates well with the concentration of antibody present, and so can give an indication of the host's humoral response. The absorbed ELISA test also has the highest sensitivity of the three serology-based methods.

The host's cell mediated immune response can be evaluated by two methods. The first is intradermal testing, which is an *in-vivo* test of immunity (Bendixen, 1978). This test is very infrequently used because of difficulties in interpretation, and poor specificity. More recently, an assay of cell-mediated immunity has been developed based on the elaboration of interferon-γ (IFNγ) by lymphocytes in response to stimulation with *Map* antigen (Billman-Jacobe *et al.,* 1992). This assay was originally developed for use in the diagnosis of tuberculosis in cattle (Rothel *et al.,*

1990), but has subsequently been modified for use in paratuberculosis, and shown to be effective in detection of ovine IFNγ (Rothel *et al.,* 1990; Burrells *et al.,* 1995). The distinct advantage of this test is that it is the only one capable of detecting the earliest stages of infection, and provides a quantitative assessment of the host's cell-mediated immunity. The test can be performed on whole blood. The limitations of this test in the diagnosis of paratuberculosis are in the difficulty of handling the blood samples quickly and with care to prevent deterioration of the cells, and that some animals in advanced stages of the disease may show poor IFNγ responses.

1.3.5 Treatment and control

Chemotherapy of paratuberculosis cases is, in general, not undertaken. The organism is sensitive *in vitro* to a number of antibiotics, but treatment is neither practically nor financially viable (reviewed by Cocito *et al.,* 1994). Control is normally based on diagnosis and removal of infected animals. The existence of subclinical cases which are excretors of high numbers of organisms complicates control measures, and consequently it is of paramount importance to diagnose infection and remove as many affected animals as possible from the group. In addition to removal of paratuberculosis cases, a number of 'common sense' hygiene measures are advocated, such as removal of neonatal calves to a clean enclosure and not allowing sucking of the dam (Chiodini *et al.,* 1984). Furthermore, care should be taken to avoid access of calves to manure from adult animals, soiled pastures, stagnant water and so forth. Due to differences in husbandry practices, such hygiene measures are likely to be more problematic in implementation when considering sheep and goats.

Vaccination is available for the control of paratuberculosis, and has been used both in cattle and in sheep (Cranwell, 1993). In the United Kingdom, use of the

vaccine is controlled by the state's veterinary services, largely due to the implications of cross reactivity in intradermal tuberculin testing. Vaccination is used in herds and flocks in which paratuberculosis is endemic as a means of reducing infection, and not in prophylaxis for uninfected groups.

1.4 ASPECTS OF RUMINANT IMMUNOLOGY

1.4.1 T lymphocytes

T lymphocytes can be divided into three major phenotypic subsets on the basis of T-cell receptor (TCR) and of coreceptor molecules which associate with MHC (major histocompatibility) molecules on antigen presentation (Janeway, 1992). The first division is on the basis of TCR, and cells are characterised by the presence of either an $\alpha\beta$ or a $\gamma\delta$ TCR. Secondly, these groups can be subclassified on the basis of the coreceptor molecules CD4 and CD8. CD4 and CD8 are usually co-expressed with the $\alpha\beta$ TCR. Interestingly, the presence of these coreceptor molecules correlates well with the function of the cell which expresses them (Janeway, 1992), and $CD4^+$ cells are considered to be helper (Th) cells whereas $CD8^+$ cells are considered to be cytotoxic (Tc) or suppressor (Ts) cells. T cells have been further subclassified on the basis of function, and on patterns of cytokine elaboration. This was first described for murine Th cells (Mosmann *et al.,* 1986), and subsequently it is in this species that T cell function has been best delineated (reviewed by Mosmann and Sad, 1996). In brief, Th cells have been shown to conform to a pattern of cytokine secretion which is characterised by production of IFNγ, interleukin (IL)-2 and tumour necrosis factor (TNF)β in clones designated Th1 (or type 1), and of IL-4 and IL-5 in clones designated Th2 (or type 2). The function of these subsets is for the promotion of cell-mediated and antibody responses respectively.

1.4.2 Gamma-delta TCR$^+$ T cells

The ovine γδ TCR was first characterised by Mackay *et al.* (1989) using the '86D' monoclonal antibody (MAb) which recognises the δ chain of the molecule. 86D precipitates a 70-75 000 MW molecule under non-reducing conditions. The ovine γδ TCR is encoded by a multiple gene loci (Walker *et al.*, 1994). Ovine γδTCR+ cells coexpress the T19 molecule (Mackay *et al.*, 1989) which is present on 90% of mature ovine γδTCR$^+$ cells (Hein and Mackay, 1991). T19 (termed WC1 in cattle) was first described by Mackay *et al.* (1986) and is a cell-surface molecule which is highly conserved in all domestic and wild ruminants (Hein *et al.*, 1991). T19$^+$ cells have been shown to recirculate differently from other T cells and are thought not to enter MLN directly from the circulation, but to enter tissues. T19 has been implicated in tissue-homing specificity and is largely absent on γδTCR$^+$ intraepithelial lymphocytes (IEL), suggesting difference in function, homing or degree of development for these cells (Gyorffy *et al.*, 1992). High concentrations of γδ TCR$^+$ cells have been found in the intestinal mucosa (Mackay *et al.*, 1989) and the gut epithelium in other species, notably chickens (Bucy *et al.*, 1988), humans (Bucy *et al.*, 1989) and mice (Goodman and Lefrancois, 1988). Gamma-delta T cells are known to be activated in an antigen-specific manner in response to bacterial, and mycobacterial infections (reviewed by Kaufmann, 1993). Compared with data available for αβ TCR$^+$ lymphocytes, there persists limited information on the antigen specificity and recognition, and function of γδ TCR$^+$ cells. A particular feature of the ruminant immune system is the prominence of γδ TCR$^+$ T cells, and indeed this is the predominant lymphocyte subset in neonatal and juvenile ruminants (Hein and Mackay, 1991), although this subset gradually decreases in relative proportion as the animals mature. Nonetheless, γδ TCR$^+$ cells remain a major subset in adult ruminant species. γδ TCR$^+$ cells are therefore likely to play an important and major role in the pathogenesis and immune response to *Map* infection. Chiodini and Davis (1992) suggested that γδ TCR$^+$ cells did not require

accessory or antigen presenting cells for tritiated thymidine incorporation in *Map* antigen proliferation assays and as such speculated that MHC does not play an essential role in antigen presentation to these cells.

1.4.3 The CD4 molecule

The ovine CD4 molecule is a single chain 56 000 MW glycoprotein present on the cell surface of the T cell subset responsible for helper/inducer functions, and acts as a receptor for MHCII and as a coreceptor for the αβ TCR in T cell activation (Hopkins et al., 1993b; Swain, 1983). Ovine CD4 was originally characterised using the monoclonal antibody (MAb) SBUT4 (44-97) by Maddox *et al.* (1985).

1.4.4 The CD8 molecule

Ovine CD8 is a dimeric glycoprotein comprising two chains, α & β, of 35 000 and 33 000 MW respectively, and is present on the cell surface of cytotoxic/suppressor T lymphocytes. In addition, homodimeric (α/α) forms are found (Maddox *et al.,* 1985) and these may be more frequent in the mucosal compartments, however, to the author's knowledge there is still no published evidence of specificity for either chain by MAbs reactive against ovine CD8 (Hopkins *et al.,* 1993b). CD8-expressing cells recognise antigen presented in association with MHCI (Swain, 1983). Many murine IEL express the homodimeric form of the CD8 molecule in contrast to the heterodimeric form found in other lymphoid tissues.(Guy-Grand and Vassalli, 1993). Ovine CD8 was originally characterised using the MAb SBUT8 (38-65) by Maddox *et al.* (1985).

1.4.5 B cells

In sheep, the ileal Peyer's patch appears to be a major organ responsible for production of B cells, in addition to the bone marrow, and indeed, the role of the Peyer's patch is one of an important secondary lymphoid organ (reviewed by Griebel and Hein, 1996). In young lambs the Peyer's patch contains approximately 70% of the total B cell population, and is the primary source of surface immunoglobulin $(sIg)^+$ B cells. It is at its greatest size in the ileum at 2-3 months after birth when it is in the form of a continuous lymphoid organ which involutes progressively thereafter until the continuous PP has disappeared by 18 months of age. B cells can recognise antigen directly by virtue of a diverse repertoire of sIg antigen receptors (Reynaud *et al.,* 1991). In sheep, sIg^+ cells make up a higher percentage of PBL than in humans (approximately 30% in sheep compared with 10% in humans) (Mackay, 1988).

1.5 IMMUNITY TO MYCOBACTERIAL DISEASES

1.5.1 Intracellular bacterial infections

Immune responses to *Map* infection have not been extensively studied. However, the immunology and immunopathology of diseases caused by related members of the genus *Mycobacterium* have been more thoroughly, although far from exhaustively, characterised. Tuberculosis is the index mycobacteriosis, against which others are compared, probably a result of its status as a human pathogen of primary importance, with high prevalence, morbidity and mortality. It has been estimated that 1/3 to 1/2 of the world's population are infected with *M. tuberculosis*, with an estimated 60 million sufferers. Globally, disease caused by *M. tuberculosis* is responsible for more deaths than any other pathogen, with in excess of 3 million

people dying of this disease annually (Kaufmann, 1993), and the World Health Organisation recently predicted that this will rise to 4 million by the year 2005 (Fenton and Vermeulen, 1996). The HIV and AIDS pandemic has been associated with a resurgence of opportunistic mycobacterial infections, notably tuberculosis and disease associated with members of the MAC (reviewed in Bloom and Murray, 1992, and Daborn and Grange, 1993). As a consequence, tuberculosis is in effect a re-emergent disease, and continues to be the subject of much research. The purpose of the following section is to review in brief some of the current facts and hypotheses concerning the immune responses to mycobacteria, and to intracellular bacterial pathogens in general.

Intracellular bacterial infections (reviewed by Kaufmann, 1993) are generally characterised to some extent by the four following points. Firstly, they exploit the intracellular habitat in order to survive and avoid the host's humoral responses, but the organisms' protein antigens are processed by the host cell and are presented to T lymphocytes. Secondly, while lymphocytes play a role in immunity and resistance, they also are involved in the pathogenesis of the disease. Thus pathogenesis is not only due to the pathogen's virulence factors, but also to the response of the host. Thirdly, tissue reactions to intracellular pathogens are typically granulomatous, whereas, extracellular microbes tend to result in purulent reactions through the mediation of polymorphonuclear leukocytes. Lastly, sterile elimination of the pathogen often fails to occur, and so chronic infection is a frequent finding of diseases associated with intracellular pathogens.

1.5.2 Interaction between macrophages and T cells

The interaction between the macrophage as both infected cell and as antigen processor/presenter and the T cell is crucial in the development of the immune response, and the eradication of the intracellular pathogen via stimulation of a DTH type response. Macrophages present antigen in association with MHC molecules (either MHC class I or class II) to T cells. T-cell production of the cytokine IFNγ results in upregulation of intracellular killing by the infected macrophage (Nathan *et al.,* 1983). Conversely, this can be reversed by the T lymphokine IL-4 (Lehn *et al.,* 1989). A Th2 type of response may follow an initial Th1 response in order to dampen the resultant DTH reaction, and thereby limit tissue inflammation and destruction (Orme *et al.,* 1993). Production of IL-12 by macrophages may be a major factor in the initiation and promotion of a type 1 response by lymphocytes, and this cytokine has been shown to be a potent driving force in the development of DTH (Trinchieri and Scott, 1995). In contrast, macrophages are also likely to contribute to damping down the DTH response in favour of a Th2 type by production of IL-10 (Gong *et al.,* 1996).

1.5.3 Tuberculosis

M. tuberculosis is a primary pathogen of humans, and is closely related to the cattle form *M. bovis*. These two organisms share similar pathology and immunopathology, and therefore the host presumably employs similar immune mechanisms in response to infection (Lucas, 1988). Tuberculosis is associated with granuloma formation, the primary organs affected being the lungs and mediastinal lymph nodes, although disseminated forms of the disease are seen in some advanced infections and in immunosuppressed individuals.

The immune responses to tuberculosis have been reviewed recently by Ehlers *et al.*, (1994); Orme *et al.*, (1993); Rook *et al.*, (1991); and Kaufmann, (1993). In brief, *M. tuberculosis* bacilli infect macrophages in which the bacilli appear to be able to persist and evade the intracellular killing mechanisms of the host cell, until the host is able to mount an adequate DTH response by which to kill the infected macrophages.

M. tuberculosis is able to persist in the hostile intracellular environment of the macrophage, even in the face of a competent T cell immune response, and appears to employ a number of mechanisms to achieve this persistence (reviewed by Britton *et al.*, 1994). Initial uptake of mycobacteria by the macrophage is by phagocytosis, and is Fc receptor independent, apparently via complement receptors, and so avoids triggering an oxidative burst by the host cell. *M. tuberculosis* grow in an intraphagosomal environment, from which the parasite appears to be able to inhibit lysosome-phagosome fusion. Virulent *M. tuberculosis* also appear to be able to escape into the cytoplasm. On exposure to mycobacteria, macrophages produce cytokines which can enhance non antigen-specific responses. Recruitment of T cells, and subsequent elaboration T cell cytokines such as IFNγ, results in upregulation of intracellular killing processes including the production of reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI). At this stage the pathogen also appears to be able to intervene by inhibition of production and by scavenging of ROI and RNI by mycobacterial cellular components, particularly lipoarabinomannan (LAM). The structure of LAM varies between mycobacterial species and strain, and is responsible for various key actions in evasion, such as blocking signal transduction pathways which result in macrophage activation and respiratory burst, and by blocking transcription of IFNγ-dependent genes such as MHCII. Different LAMs are also able to differentially induce production of TNFα by the infected macrophage, and so influence the efficacy of the host-cell's response, and this would appear to affect strain virulence. Other cell wall glycolipids have also been

implicated in these actions (Britton *et al.,* 1994). Host factors also appear to play a role in the survival of mycobacteria, notably the differences in expression of the *Bcg* gene confers resistance or susceptibility in mice (reviewed by Skamene, 1989).

As would perhaps be expected, $CD4^+$ cells appear to be the main lymphocyte subset in mediating protection against *M. tuberculosis* infection (Barnes *et al.*, 1989), both in production of IFNγ and also in a cytolytic role (Orme *et al.*, 1992; and reviewed by Orme, 1993). IFNγ appears to be the cytokine which is ultimately responsible for conferral of protective immunity to intracellular bacteria, and can be produced by $CD4^+$, $CD8^+$, γδ TCR^+ and natural killer (NK) cell mediators (Kaufmann, 1993). Analysis of cytokine production in human *M. tuberculosis*-reactive T-cell clones found that cells were producing a range of cytokines, not only Th1 cytokines, but also IL-10 and TNFα, perhaps suggesting a Th0 profile (Barnes *et al.*, 1993). The predominant cytokines elaborated by these T-cell clones were in fact IFNγ and TNFα, and these cytokines may act in a synergistic manner resulting in bacteriostasis of intracellular *M. tuberculosis* (Flesch and Kaufmann, 1990), and a role can be implied in granuloma formation. $CD8^+$ cells appear to be crucial in protection against *M. tuberculosis* infection. Flynn *et al.*, (1992), described an inability of β2 microglobulin gene knockout (β2m-/-) mice, which lack functional $CD8^+$ T cells, to resist infection with *M. tuberculosis*, with 70% of knockout mice dying within the first 6 weeks post infection. Indeed prior vaccination of β2m-/- mice with avirulent BCG (Bacille Calmette Guérin) did not protect from death. Granuloma formation, however, was evident in the gene knockout mice as well as in controls, but only β2m-/- mice had evidence of caseous necrosis. How MHCI-restricted $CD8^+$ cells are presented mycobacterial antigen is unclear, as exogenous peptide antigen is normally considered to be processed through the endocytic pathway in association with MHCII. However, exogenous antigen has been shown to access the MHCI processing and presentation pathway, (Pfeifer *et al.*, 1993), and *M. tuberculosis* has

been found free in the cytoplasm of host cells (Britton *et al.*, 1994), permitting antigen presentation through the MHCI pathway. It is also possible that some mycobacterial antigen may be processed through the cytoplasmic route, perhaps by means of a "leaky" endosomal compartment, and expressed in conjunction with MHCI. Gamma-delta T cells are also known to play a role in the immune response to mycobacteria, in particular in the early response, while αβ TCR^+ cells are crucial for protection in established disease, with studies of *M. tuberculosis* infection in αβ TCR knockout mice resulting in dissemination of infection and subsequent death (Ladel *et al.*, 1995). Gamma-delta T cells have been shown to respond by clonal expansion to mycobacterial antigen (Kabelitz *et al.,* 1990), to react to mycobacterial stress proteins (Haregewoin, *et al.*, 1989), and also to respond to whole mycobacteria in a non MHC restricted fashion (Boom *et al.*, 1992). In addition, CD1b-restricted presentation of mycolic acids and LAM to αβ TCR^+ T-cell clones which are $CD4^-$ $CD8^-$ has been described (Sieling *et al.,* 1995).

Granuloma formation in tuberculosis is intrinsic to the response to the pathogen, and the efficacy of this process dictates the outcome and duration of the infection (reviewed by Kaufmann, 1993). Different forms of granuloma have been distinguished. Productive granulomas develop under the influence of T-cell mediation. In the initial phase, T-cell-independent mechanisms (IFNγ, TNFα, IL-1, and IL-8) appear to play a role in the attraction of phagocytes, promotion of inflammation, and reduction of bacterial load. Recruitment of T cells and activated macrophages results in the formation of granulomas the purpose of which is to permit phagocytosis of bacilli liberated during lysis of the macrophage hosts by cytotoxic T cells. Cell phenotype has been related to immunopathology and granuloma formation in tuberculosis. $CD4^+$ T cells predominate, provide help for tuberculostasis by production of IFNγ and TNFα, and attract further monocytes by virtue of these and other cytokines. Lysis of infected macrophages occurs allowing phagocytosis by competent mononuclear phagocytes.

Fibrosis and calcification of the granuloma further aids containment of infection, however some mycobacteria may persist in the lesion, and this is compounded if caseation occurs, permitting growth of bacilli extracellularly in the caseous centre of the lesion. Alternatively, the granuloma may undergo necrosis, and lysis of macrophages may result in death of the organisms as a result of oxygen starvation, but may be even further injurious to the host tissue. However, the caseous centre of the granuloma may liquefy and provide a growth medium for tubercle bacilli, which, if the tubercle ruptures, can escape and permit dissemination of infection to surrounding tissues, or into the atmosphere as infective organisms. In HIV-positive patients with low $CD4^+$ cell counts, "lepromatous" type lesions have been shown to occur with foamy macrophages and necrosis present, whereas in patients with higher $CD4^+$ cell counts epithelioid lesions with giant cells were present. Lepromatous type lesions were thought to occur as a result of inadequate T-cell activation or suppression of $CD4^+$ function, and $CD4^+$ cells within lepromatous lesions did not express CD25 (Ehlers *et al.*, 1994).

1.5.4 Leprosy

M. leprae is the causative agent of leprosy and remains a significant pathogen and a major health problem worldwide because of the deformities it causes, and the social stigma with which it is still associated (reviewed by Sansonetti and Lagrange, 1981). There are still no readily available animal models for the study of leprosy (with the exception of the armadillo and gene-deficient mice), and so most of the data on the immunology of leprosy are gleaned from human patients. *M. leprae* is a very slow-growing obligate intracellular pathogen, which has a tropism for cooler areas of the body, such as skin, peripheral nerves, and nasal and oral mucosa. The different tissue predilection alone probably dictates that the pathology associated with this disease

should be different from that of tuberculosis. A spectrum of lesions is observed in leprosy, and these correlate well with a spectrum of immune responses. The disease provides an interesting study for the interaction of host and pathogen, and the balance between immunity and immunopathology. This is further complicated by the fact that leprosy is neither a static disease nor one with a linear progression, but is characterised by lesions which may fluctuate in nature, and an array of immunopathology due to the occurrence of a number of immune reactions. The immune reactions of leprosy are associated with considerable injury to the tissues, and are due to underlying immunological mechanisms. The spectrum of lesions in leprosy has long been recognised, and was definitively classified by Ridley and Jopling, (1966). The disease is associated with two polar forms, designated lepromatous and tuberculoid. In lepromatous cases, disease is severe and widespread, with considerable bacillary burdens in the tissues, and persistent bacteraemia, however this form is paradoxically associated with less tissue damage (reviewed by Sansonetti and Lagrange, 1981). Tuberculoid leprosy, on the other hand, is less diffuse, being associated primarily with cutaneous tissue and nerves. In contrast to lepromatous forms, the cellular immune responses are often excessive, and result in considerable tissue inflammation and damage, in particular in cutaneous granulomas and lesions of peripheral neuritis. Intermediate or borderline forms are common with lesions and immune reactions between the two polar forms. Reactional states occur during the progression of the disease. The first is the type 1, or reversal, reaction which is characterised by an upgrading of the cell-mediated immune response, often in response to chemotherapy, and is associated with granuloma formation with influx of immune cells (Modlin *et al.,* 1983). The second reaction is termed erythema nodosum leprosum (ENL) or the type 2 reaction, and is characterised by development of erythematous nodules, which are painful and associated with systemic illness. The mechanism underlying this reaction is thought to be the deposition of antigen-antibody complexes in the tissues, and is again linked with the commencement of chemotherapy (Modlin *et al.,* 1985). The

pathogen is attributed with a number properties which may affect the immune response, including the ability to suppress macrophages and T lymphocytes by virtue of cell-wall glycolipids (Mehra *et al.,* 1984; Moura and Mariano, 1996). *M. leprae* is also able to persist in the cytoplasm of host macrophages, and its tropism for non-professional phagocytic Schwann cells aids long-term survival in the host.

Leprosy appears to be a more consistent model than tuberculosis for paratuberculosis, and the pathological classification for leprosy (Ridley and Jopling, 1966) has been extended in the broadest terms to lesions of paratuberculosis, which have been termed lepromatous and tuberculoid (Buergelt *et al.,* 1978; Chiodini *et al.,* 1984).

1.6 ASPECTS OF GUT IMMUNOLOGY

1.6.1 The mucosal immune system

Epithelial surfaces of the body are the sites which are most likely to encounter foreign antigen and pathogenic organisms. This is true for no organ more than for the gut, which encounters a battery of antigen right from birth and continuously throughout life. Antigen can be of food origin, or in the form of microorganisms. It is therefore of fundamental importance that the host can mount a self-preserving immune response to potential pathogens, and yet develop and maintain tolerance to harmless ingested antigen. The internal milieu is separated from this vast amount of antigen by a single-cell epithelial layer, through which the nutrients required for life must be absorbed. It is little wonder that animals have developed a complex network of immune structures and functions in this organ. Non-specific defence mechanisms include a range of digestive enzymes and gastric acid in the luminal contents, and the

secretion of mucus by the mucosal surface. More specific defences are the production of immunoglobulin, and the gut-associated effector cells and organs. Discrete, organised lymphoid tissue of the gut was described by Peyer as early as 1677 (Griebel and Hein, 1996). Gut-associated lymphoid tissue (GALT) is classified as comprising the discrete, organised lymphoid tissues of the gut, including the Peyer's patches, and lymphoid nodules. MLN, IEL, and lamina propria lymphocytes (LPL), although not traditionally considered to be constituent parts of the discrete GALT are included in this context. Gut-associated lymphocytes comprise the major percentage of the total lymphocyte population (MacDonald and Spencer, 1994). IEL appear to be well characterised, whereas relatively little is published on the cellular constituents of the lamina propria. The intestinal epithelial cells (EC) themselves also have the ability to express MHCII, and experiments have shown that co-culture of MHCII positive EC and $CD8^+$ cells can result in the expansion of the $CD8^+$ population, and cytokine production by the EC (Panja *et al.*, 1995).

1.6.2 IEL

Although the presence of lymphocytes within the gut epithelium has long been recognised (Fichtelius, 1968), the function of these cells is still not completely understood. They have been quantified and characterised to represent a large, phenotypically heterogeneous population situated in a location which makes them potentially important as a first line of immune-cellular defence at a portal of entry for many pathological organisms into the body, and in a site which is exposed to a large amount of antigen. Cell-mediated defence at the mucosal surface, and surveillance of epithelial cell integrity have been suggested as roles for IEL (Janeway *et al.,* 1988). Expression of CD8 by the majority of IEL has been noted (Parrott *et al.,* 1983) and co-expression of γδ TCR and CD8 has been observed on murine IEL (Goodman and

Lefrancois, 1988). Indeed the IEL population is not a uniform one, as differences in phenotype have been noted between small-intestinal and large-intestinal murine IEL, with predominance of $\alpha\beta$ TCR^+ cells in large-intestinal IEL and higher numbers of $CD4^+$ cells present (Camerini et al., 1993). Higher densities of IEL are present in small than large intestine (Beagley et al 1995).

1.6.3 LPL

The lamina propria (LP) itself is a loosely woven matrix of connective tissue and consists of fibroblasts, capillaries, lymphatics and smooth muscle cells, and is situated between the intestinal epithelium and the basal muscularis mucosae. The LP is a highly cellular tissue with the main constituents being cells of lymphoid and myeloid origin. Ovine LPL recirculate between blood and the LP, by draining via the intestinal lymph through the MLN, enter the peripheral circulation at the level of the thoracic duct, and return to the LP by extravasation from the capillaries (Cahill *et al.,* 1977; Mackay *et al.,* 1992). Ovine LPL of memory T-cell phenotype are highly tissue specific, as cells collected from afferent intestinal lymph were labelled, recirculated, and found to home to the gut, and in contrast, these cells migrated poorly through skin (Mackay *et al.*, 1992). Unlike naive cells, LPL are L-selectin negative (Mackay *et al.*, 1992). Most IEL originate from the LP, and indeed some LPL may be in transit to the epithelium, but there is evidence to support the intestinal epithelium as an organ of lymphopoiesis (Guy-Grand and Vassalli, 1991; Poussier and Julius, 1994). Most LPL appear to be memory/effector lymphocytes which have undergone prior antigen activation, and several workers have described poor proliferation by LPL, but the ability to produce an array of cytokines in response to antigenic stimulation (Zeitz *et al.*, 1991). James *et al.*, (1986) described an increase in percentages of T cells of helper/inducer phenotype in inflammatory bowel disease.

1.7 AIMS OF THIS THESIS

The primary aims of this thesis are to examine the varied pathological manifestations of paratuberculosis and to relate these to the immune responses observed in the clinical cases of the disease. It is hoped to achieve a broad view of ovine paratuberculosis in relation to lesions observed, cellular populations at the primary site of infection and associated tissues, and the cellular and humoral immune responses. In addition, characterisation of the lymphocyte subsets in the ileum of normal sheep will be undertaken in order to provide the basis for comparison with clinical cases of paratuberculosis. It is hoped that the results of these preliminary studies, which are fundamental in nature, will be assessed in order to provide a basis for further investigation of the pathogenesis of paratuberculosis, and may ultimately lead us some way towards filling in the many gaps that are present in our current knowledge of the pathogenesis of this significant disease of ruminants.

CHAPTER TWO

GROSS AND HISTOLOGICAL PATHOLOGY IN PARATUBERCULOSIS OF SHEEP

2.1 INTRODUCTION

The gross and histological changes in *Map* infection in sheep have been descriptively reported by a number of investigators (Stamp and Watt, 1954; Rajya and Singh, 1961; Nisbet *et al.,* 1962; Reddy *et al.*, 1984; Carrigan and Seaman, 1990; Pérez *et al.*, 1996). In addition the lesions of paratuberculosis in cattle (Buergelt *et al.*, 1978), and North American wild ruminants (Williams *et al.,* 1983) have been described. Pathological changes have been reviewed by Chiodini *et al.,* (1984), and by Barker *et al.,* (1993).

The initial clinical examination of animals affected by Johne's disease reveals low bodily condition score and often emaciation. Diarrhoea is frequently the clinical presenting sign in cattle, however in sheep this is considered to be less common. Peripheral, especially intermandibular, oedema may be present in advanced cases. Necropsy examination of the abdominal cavity usually reveals oedema of the serosa and mesentery, enlarged ileocaecal and mesenteric lymph nodes, dilated ileum, and prominent, dilated lymphatic vessels with a typically glassy appearance. Intestinal lesions are most frequent in the distal to terminal ileum, with the earliest lesions thought to occur at the level of the ileocaecal valve, although any part of the intestinal tract may be affected, from duodenum to rectum. In other areas of the gut lesions are found more sporadically. On opening the ileum, the mucosa is often grossly thickened, fleshy and transversely corrugated with a matt, velvet and faintly granular appearance. These transverse rugae do not flatten when the gut is stretched. Some

Map strains, in particular sheep strains, give rise to a yellow pigment which suffuses the mucosa. Different degrees of extensiveness occur with lesions being either segmental and discrete, or diffuse and coalescent along the gut. In some cases there are no macroscopic lesions suggestive of paratuberculosis, disease being confirmed by microscopic examination only. On histological examination of Ziehl-Neelsen (ZN) stained tissue, clumps of acid fast (AF) staining bacilli (AFB) are frequently seen in the macrophages, which are usually large and have distinctive foamy cytoplasm. Villus atrophy is frequently in evidence, villi being thickened, distorted, and stubby in appearance and may be coalescent. In addition, crypts often appear hyperplastic. In some areas, microgranulomatous foci can be seen and germinal centres present in the organised lymphoid tissues of the mucosa and submucosa. Giant cells may be found in both gut and MLN. Focal areas of caseous necrosis and less commonly calcification have been described, especially in secondary sites of infection such as MLN and liver, and particularly in sheep and goats. AFB can often be demonstrated in mesenteric lymph nodes and occasionally in the liver. There may be a diffuse infiltrate of lymphoid cells in addition to, or instead of, the presence of large macrophages which have abundant foamy cytoplasm and are frequently packed with organisms. The disease is reportedly more diffusely granulomatous in cattle, with the lymphoid form of cellular infiltrate infrequently described.

In ovine paratuberculosis in particular, a spectrum of histological lesions is seen varying from small focal granulomatous areas with a predominantly lymphoid cell infiltrate, low numbers of infected macrophages with low intracellular bacterial burdens, with or without the presence of giant cells (paucibacillary or tuberculoid type), to diffuse, extensively affected areas containing high numbers of large macrophages with heavy intracellular bacterial burdens and relatively few lymphocytes (multibacillary or lepromatous type) (Stamp and Watt, 1954; Rajya and Singh, 1961; Reddy *et al.*, 1984; Carrigan and Seaman, 1990; Pérez *et al.*, 1996).

Differences in lesions may be due to the mycobacterial species and/or strain involved. In *Map* infected lambs intestinal lesions tend to be diffuse in character, while *M. a. silvaticum* (*Mas*) infection results in focal caseous granulomatous lesions, particularly in the lymphoid tissues (García-Marín *et al.,* 1994). Different host species may also respond differently to *Map* infection.

The purpose of this study was to examine the range of gross and histological changes seen in this sample of the sheep population with clinical paratuberculosis in Southern Scotland and to quantify the pathological changes employing a scoring system based on that described by Carrigan and Seaman, (1990). The aim was to relate the pathological findings to a number of other observations of the host response made in these sheep: clinical signs, serological findings, gross and histological pathology and ultimately to the immunology of the infection. A range of histopathological findings has been described and the hypothesis was made that these were a result of differing pathological or immunological mechanisms. The changes were examined and scored accordingly and will be related to further studies.

2.2 MATERIALS AND METHODS

2.2.1 Sheep

For the purposes of this study, 29 normal control, and 45 diseased adult female sheep were obtained from farms in Southern Scotland (Dumfriesshire, Lanarkshire, and the Lothians). Sheep were principally of the Scottish Blackface and Cheviot breeds. Diseased sheep were selected from flocks with endemic paratuberculosis, which had been previously confirmed by pathological or microbiological examination. None of the farms had employed paratuberculosis vaccination as a method of disease

control. *Ante-mortem*, sheep were subjected to a clinical examination, and condition scored on a recognised scale of 1-5 (a score of 1 signifying emaciation, and 5 obesity). The presence of faecal soiling, or of normal, pelleted faeces was noted at this time. Whole blood was collected by percutaneous jugular venipuncture into a glass universal with no anticoagulant present, and serum later decanted and stored at -30°C until used in the AGID test (Sherman *et al.*, 1984) for serological evidence of paratuberculosis infection. All AGID tests were performed by the Scottish Agricultural Colleges Veterinary Investigation Centre, Bush Estate, Roslin.

2.2.2 Necropsy technique

Animals were euthenised by intravenous pentobarbitone sodium injection (Euthatal, Rhône Mérieux, Harlow, UK) and immediately exsanguinated by sectioning the jugular, axillary, and femoral blood vessels.

A full routine necropsy examination was performed immediately, and all gross lesions noted. Particular attention was paid to carcase condition, degree of mesenteric/serosal oedema, and the size of the mesenteric lymph nodes. Intestinal lesions were classified according to position and extent of intestinal lesions, apparent thickness of the mucosa, presence of yellow Johnin pigment, presence of mucosal corrugation and mucosal crevicing, evidence of lymphatic cording. Evidence of significant intercurrent disease resulted in exclusion from the study.

The intestinal tract was sampled by excision of 4 cm^2 samples of tissue from the proximal duodenum (5 cm from the pylorus), mid-jejunum, terminal ileum (5 and 10 cm from the ileocaecal valve), caecum, mid-spiral colon, and terminal rectum (10 cm from the anus). Samples were also taken from mesenteric and ileocaecal lymph nodes

(MLN/ICLN). In addition, samples were taken from the following organs and tissues: prescapular lymph node, spleen, liver, kidney, myocardium, lung, uterus, mammary gland, and if present, foetal tissue. Tissue samples were taken into 10% buffered formalin fixative, and routinely processed, and sections cut at a thickness of 4 μm for histological examination. In addition, fresh samples of ileum were taken for nucleic acid extraction, and the detection of *Map* specific genomic DNA (IS900) by PCR. Fresh samples were snap frozen in dry ice/isopentane slush and stored at -70°C until required.

2.2.3 Haematoxylin and eosin staining

Hamatoxylin and eosin (HE) staining was performed on a Shandon Linistainer automated staining machine (Shandon Ltd., Basingstoke, UK). Sections were initially dewaxed in a graded series of decreasing percentage alcohol dilutions. They were then immersed in Harris haematoxylin (Surgipath, St. Neots, Cambs., UK) for one minute, washed for one minute in running tap water, the haematoxylin staining 'blued' by immersion in Scott's tap water substitute for 1 minute, then immersed in eosin (Surgipath) for 30 seconds, washed in tap water for one minute, and then dehydrated through a graded series of increasing percentage alcohol dilutions before finally being cleared in xylene. The stained sections were mounted in DPX mountant (BDH, Poole, UK) and covered with glass coverslips.

2.2.4 Ziehl-Neelsen staining for acid-fast bacteria

ZN staining was performed by flooding slides with tissue sections in Carbol Fuchsin stain (BDH) and heating gently until the stain began to steam. This was

repeated up to three times, and then left to stain at room temperature for 5 minutes. Slides were then rinsed in acid-alcohol (1% HCl in 70% ethanol) until the tissue was decolourised, and then rinsed in tap water. Sections were counterstained with Harris haematoxylin, and processed as above by addition to the haematoxylin bath on the Shandon Linistainer, but omitting passage through the eosin bath. Prior to clearing in xylene, sections were rinsed in 2-ethoxyethanol (BDH), and stained with tartrazine (BDH) (saturated solution in 2-ethoxyethanol) for 15 seconds. Sections were then cleared and mounted as above.

2.2.5 Histopathological assessment of tissues

Detailed histological analyses were made of sections of ileum from both control and diseased sheep. A number of observations were made and lesions assessed by a scoring analysis by one pathologist (Dr C.J. Clarke). In this analysis lesions were assessed by degree of severity and scored on a scale of 0-3, with 0 equivalent to normal, and 3 equivalent to severe or extensive lesions. This scoring system was based on that described by Carrigan and Seaman, (1990). The observations included in this study were concerned mainly with the cell type and number present in the tissues, in particular number of lymphocytes, neutrophil polymorphs, eosinophils, large macrophages/epithelioid cells, and Langhans giant cells.

The distribution of large (infected) macrophages in the tissue was also noted on the basis of scattered, focal/nodular, or diffuse, sheet-like types of distribution. An assessment was made of the percentage of tissue macrophages containing intracellular AFB (0 - none; 1 - <20%; 2 - 20-75%; 3 - >75%). To this end, up to eight randomly-chosen fields of lamina propria were observed (at high power) and a total of two hundred macrophages examined, and percentage figures calculated. In addition, the

approximate average number of AFB in each macrophage was noted and scored (0 - none; 1 - <10; 2 - 10-60; 3 - >60).

These observations were applied to three anatomical compartments of the ileum, namely mucosa, submucosa, and serosa.

Other areas of the intestinal tract were subjected to histopathological examination in order to determine the extent of the lesions, as were sections from the other organs and tissues sampled at necropsy. Particular attention was paid to the detection of the presence of AFB and granulomata in these tissues.

2.2.6 Histometric analysis

A computer assisted image analysis system (Quantimet 500C image analysis system, Leica Instruments, Cambridge, UK) was employed to provide morphometric measurements of normal control, and diseased tissues. For this purpose, routinely processed, HE stained tissues were used. Measurements were made of the depth of each layer of the ileum using the Quantimet 500 system, which had been pre-calibrated using a calibration slide of known scale. Initial measurements were made by the author, however for the purposes of this study, data are derived from measurements made by Dr C.J. Clarke. Notwithstanding, there was a high degree of parity in the measurements of both users. Measurements were made of ileal full wall thickness (villus tip to serosa), villus height, lamina propria thickness (villus base to muscularis layer), mucosal thickness (villus height + lamina propria thickness), submucosa, muscle layer, and serosa thicknesses. For each of the above, the mean values were taken of four measurements per section. The percentage area of the lamina propria occupied by crypt glandular tissue was calculated, taking the mean area from four

random fields of lamina propria. In addition, a subjective assessment was made of the degree of villus flattening, fusion and atrophy, and scored on a scale of 0-3 as described previously (0 - normal, 3 - severe).

2.2.7 Statistics

Morphometric analysis and histological scoring data were found to be normally distributed when classified into groups ('multibacillary', 'paucibacillary', and 'control' - *vide-infra*), and accordingly were subjected to analysis of variance tests, with comparisons between two groups subsequently made by applying Student's T test. Correlations between groups were tested using the covariance of the two data sets, divided by the product of their standard deviations. Microsoft Excel for Windows, version 5.0, was used for the statistical analysis.

2.3 RESULTS

2.3.1 Normal control sheep

All control sheep were confirmed as being non-diseased on the basis of negative AGID test serology results, clinical examination, and absence of gross and microscopic lesions of paratuberculosis on necropsy and histology (table 2.1). Signs of significant intercurrent disease resulted in removal from this study.

Table 2.1: Clinical findings in normal control and diseased sheep.

Clinical Observation	*Control (n=29)*	*Multibacillary (n=31)*	*Paucibacillary (n=14)*
Condition Score (mean)	3.06	1.10	1.30
Pelleted Faeces	100	65	86
Soft Faeces	0	16	14
Diarrhoea	0	19	0
AGID test +ve	0	88	36

Table 2.2: Gross pathological findings of normal control and diseased sheep.

Necropsy finding	*Control (n=29)*	*Multibacillary (n=31)*	*Paucibacillary (n=14)*
Carcase emaciation	0	100	85
Carcase oedema	0	83	75
Ileum:			
Thickened wall	0	100	85
Mucosal pigmentation	0	90	0
Mucosal ridging	0	43	31
Mucosal crevicing	0	26	8
Lymphatic cording	0	83	71
MLN/ICLN enlargement	0	80	50

Results are expressed as percentage of each group showing signs of each observation, with the exception of mean bodily-condition score.

2.3.2 Categorisation of *Map* infected sheep

Infected sheep were initially categorised into two groups on the basis of the number of AFB observed in the ileal mucosa (table 2.4). These groups were termed the multibacillary (MB) (score of 2 or 3) and paucibacillary (PB) (score of 0 or 1) groups, and the hypothesis made that each group was characterised by different pathological lesions, and a result of different immunopathological mechanisms.

2.3.3 Clinical examination

On clinical examination, diseased sheep were found to have lower bodily condition scores than normal, control animals (table 2.1). The majority of infected sheep were considered to be extremely thin, and in most cases where a reason for removal from the flock was given, it was due to ill thrift. In addition, some sheep were noted to be moribund on examination. A small number of diseased sheep were found to have evidence of diarrhoea (19% of the multibacillary group only), and some soft faeces (15% of diseased sheep), but this has been considered not to be a consistent finding in ovine paratuberculosis, and in keeping with this, the majority had normal, pelleted faeces. AGID tests were performed on all animals and 86% of multibacillary cases found to be serologically positive compared with only 36% of paucibacillary cases (equivalent to 72% of all diseased animals).

2.3.4 Gross pathology

On necropsy examination, 95% of carcases of all diseased animals were emaciated (table 2.2), which was equivalent to 100% of multibacillary and 85% of

paucibacillary cases. Evidence of systemic carcase oedema was a frequent finding, affecting the majority of cases. Gross evidence of thickened ileal wall was found in 95% of diseased animals (figures 2.1 and 2.2), and as such proved to be the most consistent gross change in the intestinal tract. Also notable were the gross changes of mesenteric and ileocaecal lymphadenomegaly, and lymphangitis of the serosal lymphatic vessels, characterised by thickening and cording. These changes were in evidence in the majority of cases. The luminal surface of the ileal mucosa had a granular appearance, and mucosal pigmentation was noted in varying degrees, from faint yellow to bright orange, in multibacillary cases only (90%) (figure 2.2). No animal with paucibacillary lesions had visible mucosal pigmentation. Transverse corrugation of the luminal surface of the ileum into distinctive rugae was seen in some cases (43 % and 31% of MB and PB cases respectively), with mucosal crevices present in fewer animals, mostly of the multibacillary group (26%).

2.3.5 Lesion distribution

The ileum was the most consistent site of intestinal lesions (table 2.3), with histological evidence of paratuberculosis being present in every animal. In addition, all animals with the exception of a small number of the paucibacillary group (15%) had gross lesions of the ileum, which were always most distinct at the terminal end of the ileum, in proximity to the ileocaecal valve. The 15% of paucibacillary animals with no gross ileal lesions in fact showed no gross lesions of any area of the intestinal tract, in spite of the presence of clinical signs and histopathological change. The distribution of lesions appeared to spread proximally and distally along the intestine from the ileum, with jejunum and caecum being the next most consistently affected regions (table 2.3). However, very few animals had lesions of the proximal (duodenum) or very distal (rectum) areas of the intestine. Animals with soft faeces or diarrhoea always had

Figure 2.1: Intestines of sheep with paratuberculosis. Thickening of ileal wall is evident and mesentery appears oedematous with enlarged mesenteric lymph nodes.

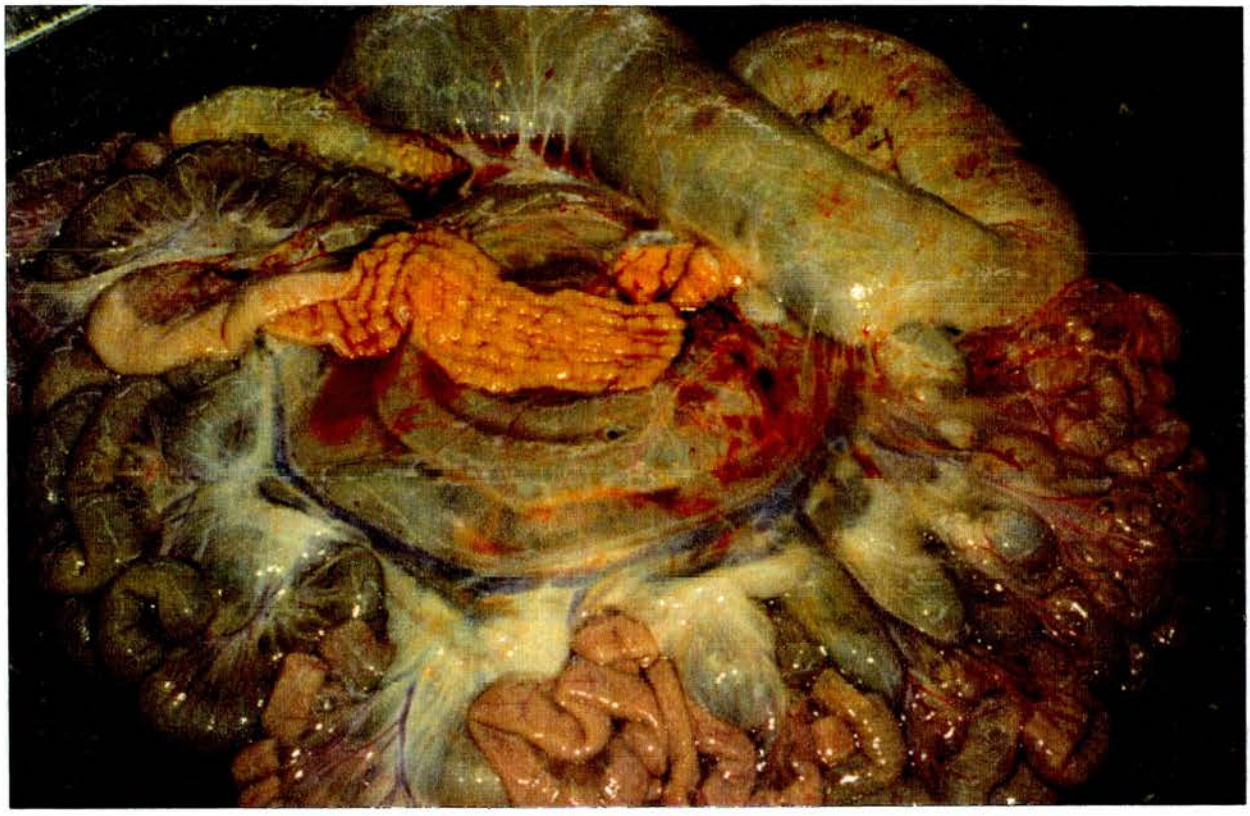


Figure 2.2: Close-up view of opened ileum. Diffuse yellow pigmentation of the mucosa is present. Luminal surface shows distinctive rugae, and crevices are evident.

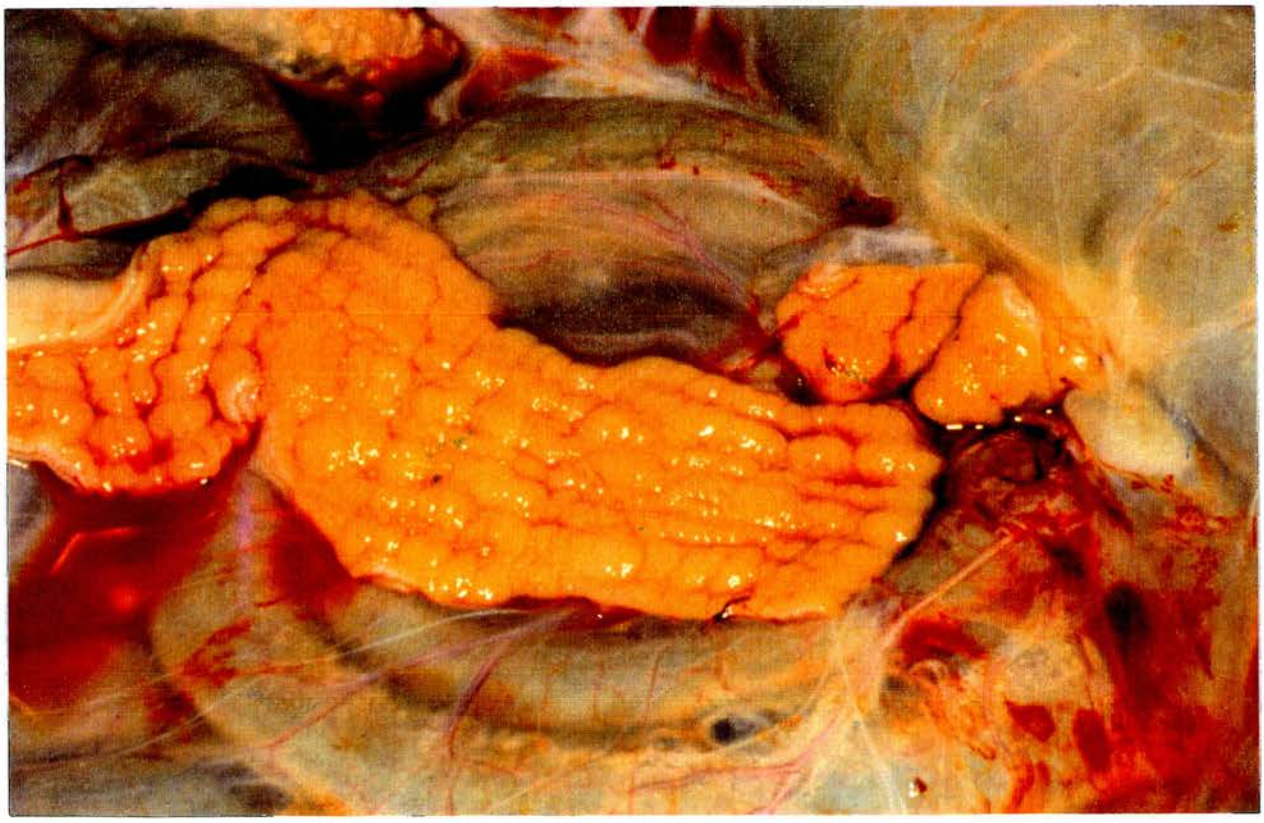


Table 2.3: Distribution of gross and histological lesions associated with *Map* infection in groups of diseased sheep; intestinal and other tissues.

Group	*Multibacillary*	*(n=31)*	*Paucibacillary*	*(n=14)*
Tissue	*Gross lesions*	*Histological lesions*	*Gross lesions*	*Histological lesions*
Duodenum	3	3	0	9
Jejunum	100	100	68	68
Ileum	100	100	85	100
Caecum	74	100	46	80
Colon	47	79	15	33
Rectum	0	25	0	0
MLN/ICLN	80	97	50	93
Liver	6	61	0	38
Lung	0	3	0	0

Results are expressed as the percentage of animals of each group found to have lesions.

lesions present in the large intestine. Histological lesions were present in greater than 90% of MLN/ICLN, with gross lymphadenomegaly being present in 80% of multibacillary cases. The multibacillary group were in general characterised by more diffuse, widespread lesions along the length of the gut. The most frequently secondarily affected organ (with the exception of the lymph nodes draining the site of infection) was the liver, which was as anticipated given its function and location in relation to the intestinal tract. Granulomatous histological lesions were present in the livers of 61% of multibacillary, and 31% of paucibacillary cases, and were in the form of small granulomata sporadically distributed within the hepatic parenchyma, and occasionally in periportal areas. One animal of the multibacillary group had peribronchial granulomata in the lungs. However, neither gross nor histological lesions were found in any of the other organs examined in these sheep.

2.3.6 Histological lesions

Neither histological evidence of cellular infiltrate, nor presence of AFB was detected in the ileum or MLN/ICLN of any control animal (figure 2.3). The histological observations and grading data of the ilea and regional LN of the diseased groups are summarised in table 2.4.

2.3.7 Histological lesions and cellular infiltrates of multibacillary cases

Ileal mucosal lesions in the multibacillary group were characterised by a marked macrophage infiltrate, with all animals having high numbers of macrophages present and all animals were scored 2 or 3, with 71% given a score of 3. The distribution of large macrophages was also characteristic (table 2.5), with distribution throughout the

Table 2.4: Histopathological findings in diseased sheep.

	Multibacillary (n=31)				*Paucibacillary (n=14)*			
Score	*0*	*1*	*2*	*3*	*0*	*1*	*2*	*3*
Ileal mucosa								
Lymphocytes	3	32	65	0	0	7	57	36
Macrophages	0	0	29	71	0	71	29	0
Neutrophils	48	48	4	0	29	64	7	0
Eosinophils	90	10	0	0	71	21	8	0
Giant cells	90	7	3	0	57	14	29	0
%MP infected	0	0	3	97	57	29	14	0
Mean AFB in MP	0	0	6	94	57	43	0	0
Submucosa								
Lymphocytes	3	78	16	3	0	50	43	7
Macrophages	6	52	16	26	14	86	0	0
Neutrophils	55	42	3	0	64	36	0	0
Eosinophils	100	0	0	0	86	14	0	0
Giant cells	97	3	0	0	86	14	0	0
%MP infected	6	16	13	65	79	21	0	0
Mean AFB in MP	6	16	36	42	86	14	0	0
Serosa								
Lymphocytes	19	81	0	0	29	71	0	0
Macrophages	61	19	13	7	64	29	7	0
MLN/ICLN								
Granulomata	3	22	53	22	7	43	29	21
Mean AFB in MP	40	33	20	7	79	14	0	7

Results are expressed as a percentage of each group for each score. Scores represent 0- normal, 1-mild, 2-moderate, and 3-severe infiltrate or change compared with normal tissues. MP = macrophage.

Table 2.5: Distribution of macrophages in ileal lesions.

	Multibacillary (n=31)			*Paucibacillary (n=14)*		
	Scattered	*Focal*	*Diffuse*	*Scattered*	*Focal*	*Diffuse*
Ileal Mucosa	0	6	94	8	71	21
Submucosa	14	45	41	31	69	0

Table 2.6: Morphometric measurements of ileum from normal control, and from diseased sheep, according to microbiological group.

Measurement	*Control (n=29)*	*Multibacillary (n=31)*	*Paucibacillary (n=14)*
Full wall thickness	1607 (49)	3302 (183)**	2482 (197)**##
Mucosa thickness	807 (26)	1100 (36)**	1074 (77)**
Villus length	535 (17)	227 (19)**	295 (74)**
Lamina propria	272 (16)	873 (36)**	829 (62)**
Submucosa	158 (19)	781 (66)**	391 (61)**##
Muscle	545 (30)	783 (56)**	752 (60)**
Serosa	87 (8)	635 (110)**	265 (70)*##
Glandular % in LP	47.4 (1.3)	24.3 (1.9)**	30.2 (2.4)**
Villus flattening/fusion score	0 (0)	2.16 (0.1)**	2.0 (0.2)**

Values are mean (SD) in μm, except for glandular percentage of LP (%), and villus flattening/fusion observation, which were scored on a scale of 0-3. (0 - absent to 3 - severe). Significant differences from the control group are denoted ** ($P<0.01$) and * ($P<0.05$). Significant differences between multibacillary and paucibacillary groups are denoted ## ($P<0.01$).

ileal mucosa being diffuse and sheet-like in 94% of cases, with multifocal aggregates being present in only 6% of cases, and no animals showing a pattern of individual, scattered macrophages. Macrophages in multibacillary cases were generally found to be large and to have abundant eosinophilic cytoplasm, which occasionally appeared vacuolated and refractile due to the presence of large numbers of intracellular bacilli (figures 2.4, 2.5 and 2.6). Macrophage cell borders were usually distinguishable, although macrophages occasionally had the appearance of true epithelioid cells within some granulomatous lesions. Lymphocytes were observed in mildly (32%) to moderately (65%) increased numbers, and were found to be spread diffusely between the macrophages. A mildly increased neutrophil polymorph (PMN) infiltrate was found in 52% of cases, and this cell type was seen both diffusely throughout the mucosa and in some inflammatory foci. Eosinophils, however, were considered to be mildly increased in only 10% of cases, with the remaining cases having normal numbers. Langhans giant cells were found infrequently, and in only 10% of multibacillary cases. In the submucosal compartment, macrophage infiltrates were also noted (figure 2.5), but in this area were found in focal aggregates, and as scattered cells (45% of cases focally, 41% diffusely distributed, and 14% scattered cells), compared with an almost exclusively diffuse, sheet-like distribution in the mucosa. A mild to moderate lymphocytic infiltrate was also found in the submucosal tissues in 97% of cases, 78% of cases being mildly affected. In addition a comparable grade and percentage of PMN to that found in the mucosal compartment (45% mild infiltrate) was noted. Eosinophil accumulations were not seen in the submucosa, and only 3% (equivalent to one animal) had giant cells present in this compartment. A mild lymphocyte infiltrate extended into the serosal layer in 81% of cases, and a mild to marked macrophage infiltrate in 39%. Granulomata were present in varying severity in 97% of MLN/ICLN, normally in cortical areas, and were small and multifocal, although large macrophages were also occasionally seen in medullary sinuses and in the sub-capsular area (figure 2.7).

Figure 2.3: Normal sheep. Ileum of control sheep showing individually distinct villi and normal mucosal structure. HE (x100).

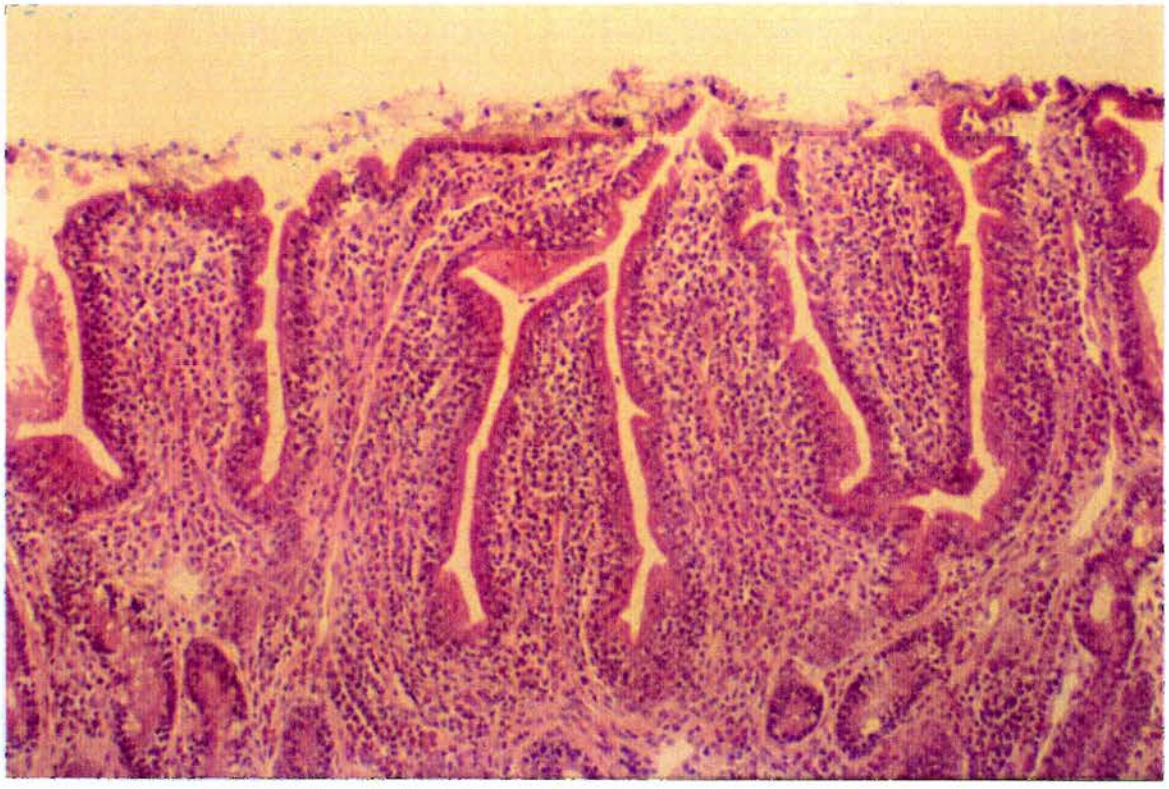


Figure 2.4: Multibacillary case (P17). Typical appearance of ileum with disruption of the normal mucosal structure and fusion of the villi, which are thickened and shortened. Lymphatic vessels (L) are dilated. Sheets of large macrophages are evident. HE staining (x100).

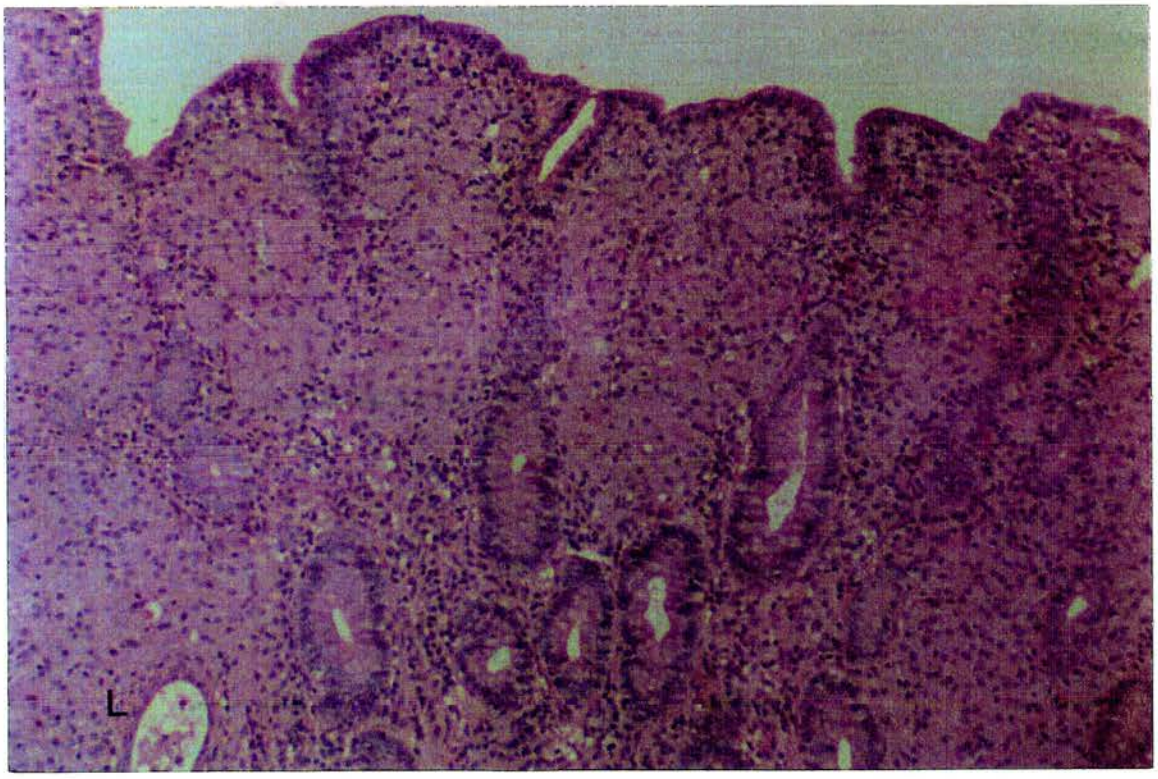


Figure 2.5: Multibacillary case (P20). Large, infected macrophages in the ileal mucosa and submucosa (S) below the muscularis mucosa (M). Amorphous eosinophilic debris is visible in the crypts (arrow). HE (x100).

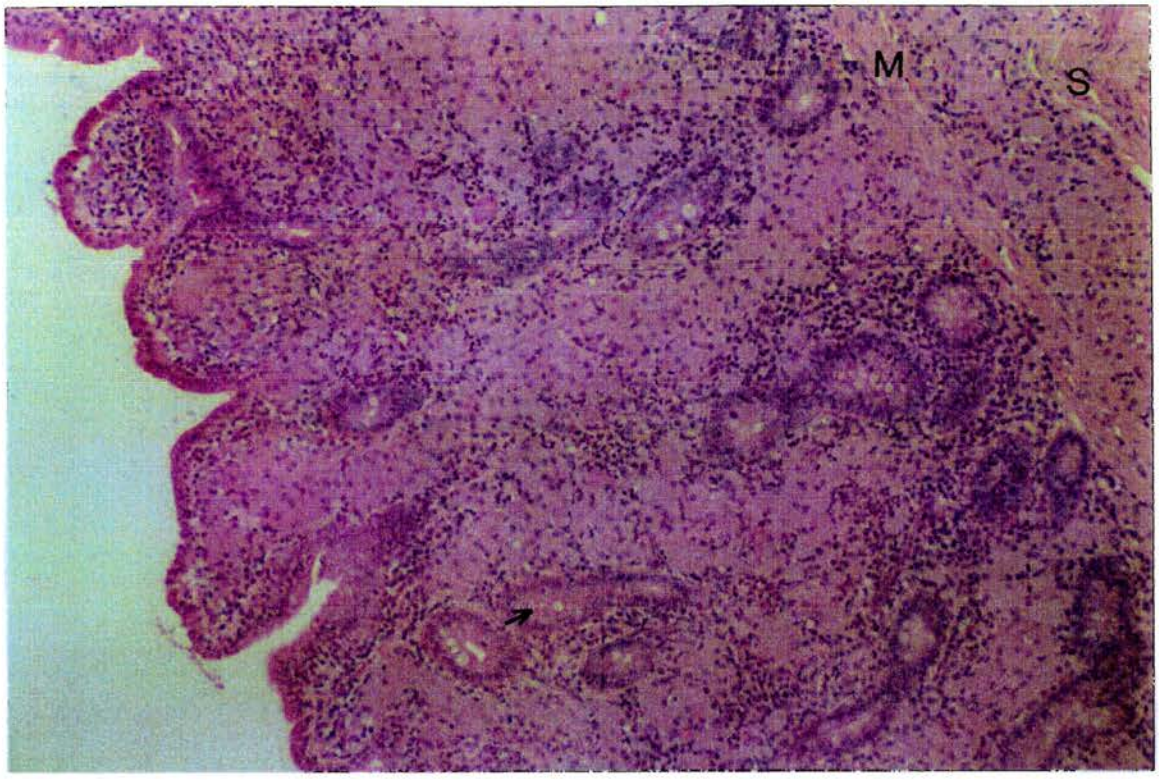


Figure 2.6: Multibacillary case. Large, infected macrophages in the ileal lamina propria. The abundant, pale, eosinophilic-staining cytoplasm appears vacuolated. HE (x400).

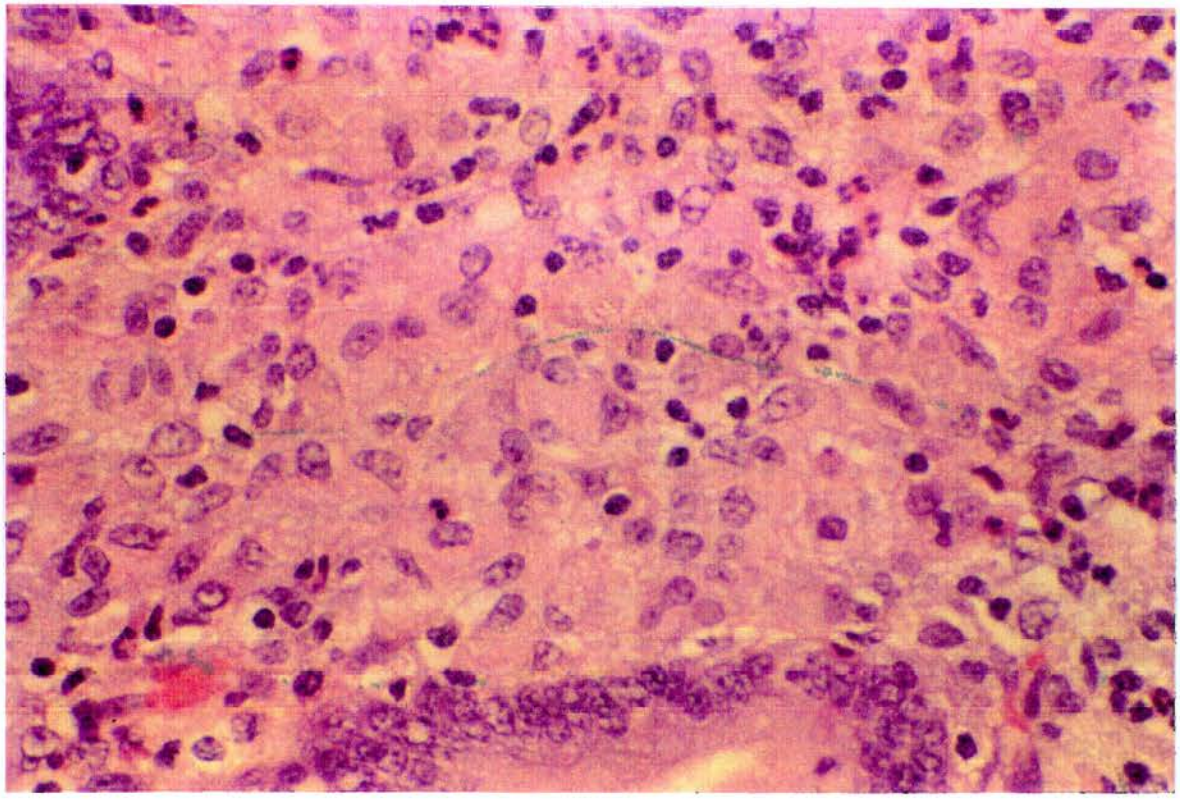


Figure 2.7: Multibacillary case (P20). Granulomatous lesions in the mesenteric lymph node. "Nests" of infected macrophages with intracellular AFB are evident in the cortical and subcapsular areas of the node. ZN (x100).

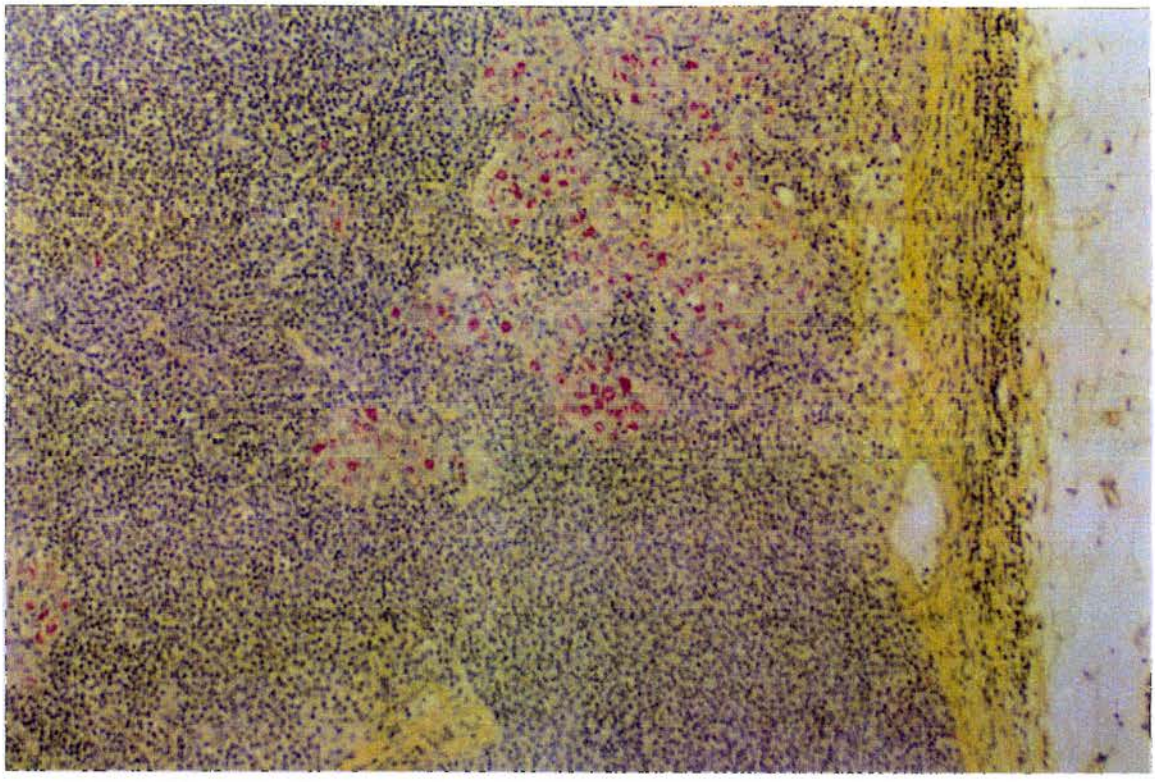
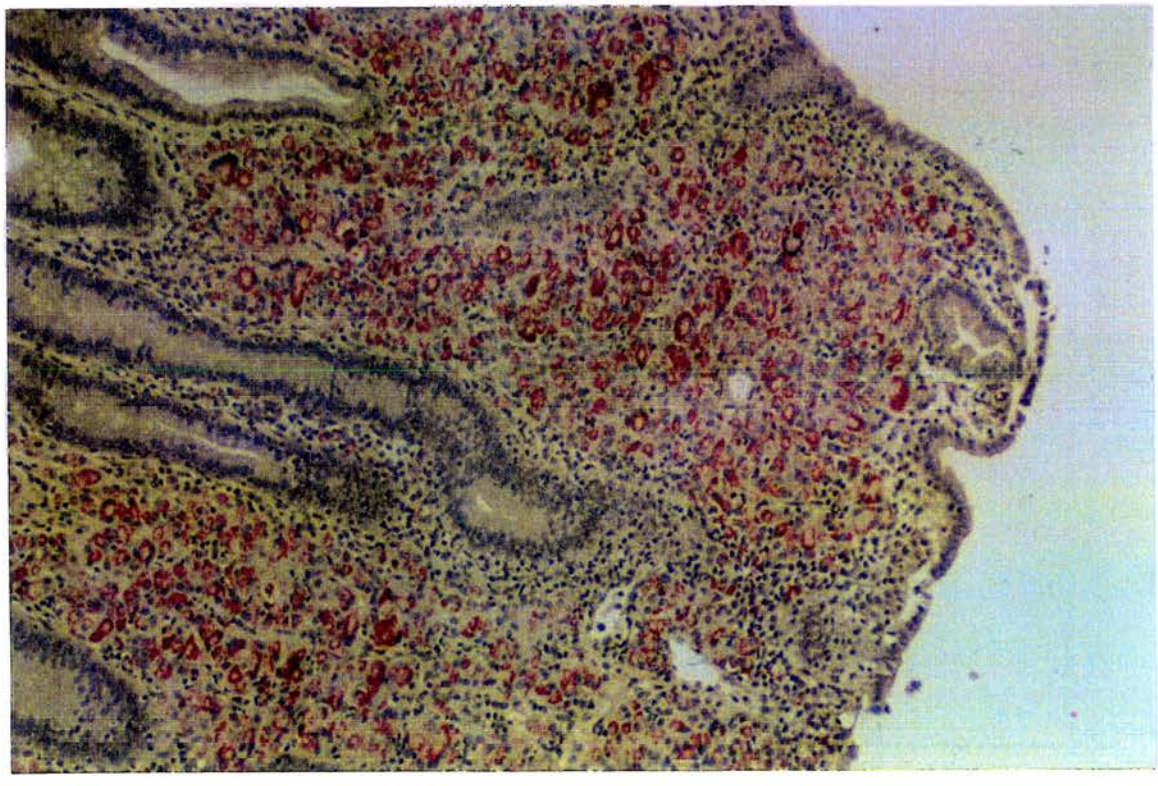


Figure 2.8: Multibacillary case (P40). Large numbers of macrophages are present in the mucosa with AFB staining pink within macrophage cytoplasm. ZN staining (x100).



As the classification implies, intracellular AFB were always present in large numbers in macrophages in the lamina propria of multibacillary cases (figure 2.8). 94% of cases had an average approximate number of AFB per macrophage in excess of sixty (figure 2.9). However, in comparison the bacillary load in the submucosal compartment was somewhat decreased (42% having greater than 60 AFB per macrophage).

Intestinal crypts often had evidence of epithelial cell hyperplasia, and were frequently occluded by amorphous eosinophilic debris (figure 2.5), with occasional small foci of necrosis. Dilated lymphatic vessels were occasionally found within the mucosa (figure 2.4).

2.3.8 Histological lesions and cellular infiltrates of paucibacillary cases

A notably different type and pattern of cellular infiltrate was present in the paucibacillary group, and was characterised in the main by increased densities of lymphoid cells instead of sheets of macrophages (tables 2.4 and 2.5). In the mucosal compartment, moderate (57%) to severe (36%) lymphocytic infiltration was in evidence in 93% of paucibacillary cases (figure 2.10). A mild (71%) to moderate (29%) large-macrophage infiltrate was present in the mucosa of all cases, however fewer macrophages were infected by AFB, and only 14% of cases had in excess of 20% of macrophages infected. Macrophage distribution was focal in the majority (71%) of cases, and only scattered macrophages were seen in 8% of cases (table 2.5). Nevertheless, diffusely distributed macrophages were still seen in 21% of cases, but tended not to be so extensive, and in addition were associated with greater densities of lymphocytes. In further contrast with the multibacillary group, Langhans giant cells

Figure 2.9: Multibacillary case. Acid fast rods packed in the cytoplasm of macrophages infiltrating the ileal mucosa. ZN staining (x400).

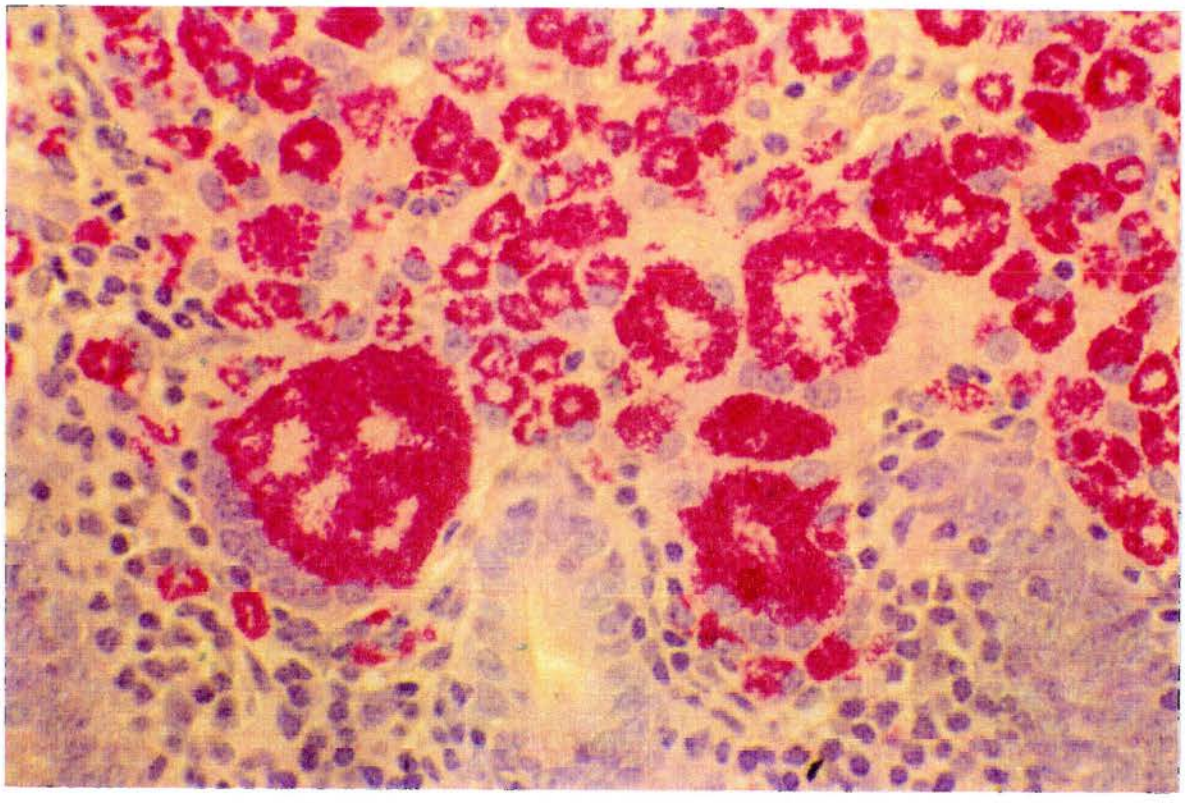
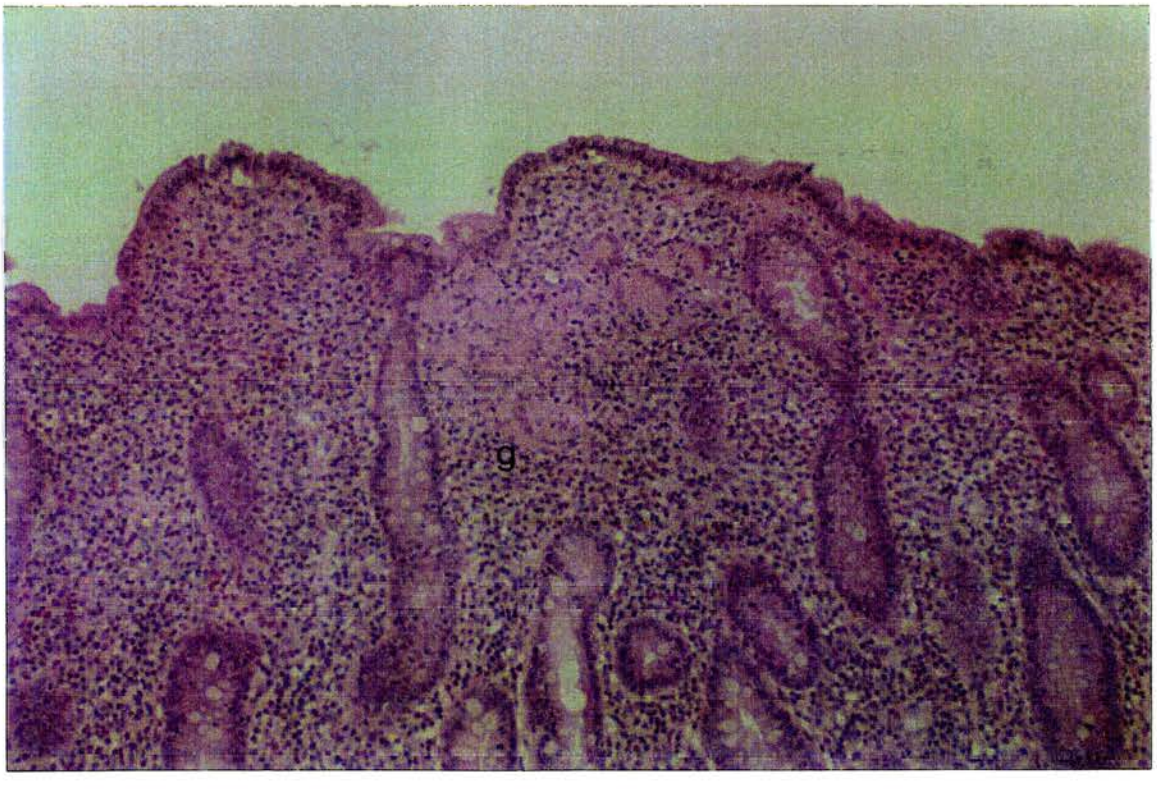


Figure 2.10: Paucibacillary case (P23). Macrophages are present in a granuloma (g) and three Langhans type multinucleate giant cells are visible. Many lymphoid cell nuclei are apparent. Villi are coalescent and normal mucosal architecture is severely disrupted. HE (x100).



were in evidence in 43% of cases (figures 2.10 and 2.11), 29% of cases having moderate numbers (compared with 3% of multibacillary cases). 71% of the paucibacillary group had mildly (64%) to moderately (7%) elevated numbers of neutrophil polymorphs, and in addition 21% had a mild and 8% a moderate eosinophil infiltrate. As in multibacillary lesions, cellular infiltrates were also seen in the submucosa (figure 2.12) and serosa (figure 2.13), and reflected the cell types present in the mucosa by appearing predominantly lymphoid in nature, with focal aggregates and scattered individual macrophages present. All animals had some degree of lymphocyte infiltrate into the submucosa, and 86% a mild macrophage infiltrate. Neutrophils and eosinophils were present in 36% and 14% of cases respectively to a mild degree only. Giant cells were detectable in the submucosa at a frequency of 14% of cases compared with 3% of the multibacillary group (figure 2.12). The serosa was affected by a mild lymphocyte infiltrate in 71% of animals, and by macrophages in 36%. Granulomata were present in the MLN/ICLN of 93% of the paucibacillary group.

Paucibacillary cases were, again as implied by the classification, associated with the presence of fewer AFB (figure 2.14). Lengthy and detailed scrutiny of ZN-stained sections of ileum revealed AFB in the macrophages of only 50% of cases, and in 86% of cases in fewer than 20% of macrophages. AFB numbers were in general fewer than 10 bacilli per macrophage.

2.3.9 Statistical observations

Highly significant correlations were found between macrophage infiltration into the ileal mucosa and the percentage of macrophages containing AFB, and the mean AFB load per macrophage ($P<0.001$). A highly significant correlation was also

Figure 2.11: Higher power magnification of paucibacillary case P23, same field as Figure 2.10, showing three Langhans type giant cells. HE (x400).

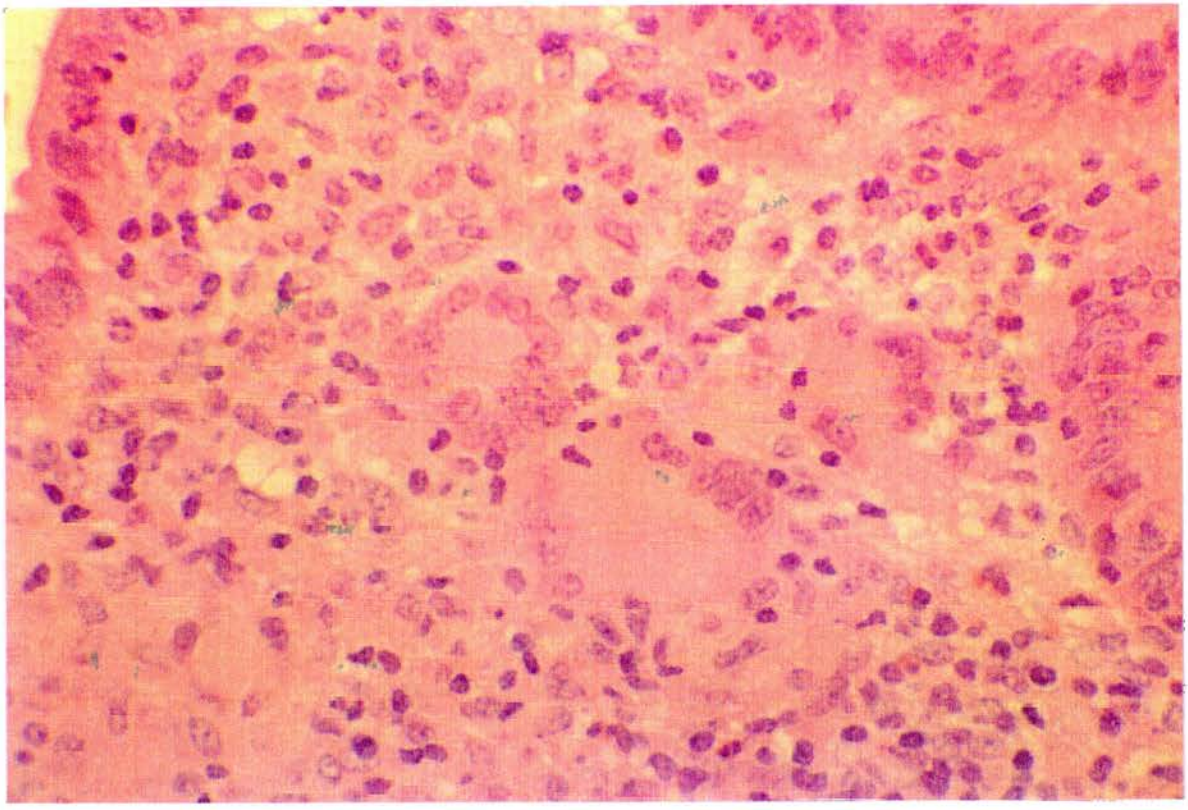


Figure 2.12: Paucibacillary case (P23). Dense lymphocytic infiltrate in the submucosal area beneath the muscularis mucosa (MM) under which focal macrophages (M) can be seen. Multinucleate giant cell (gc). HE (x100).

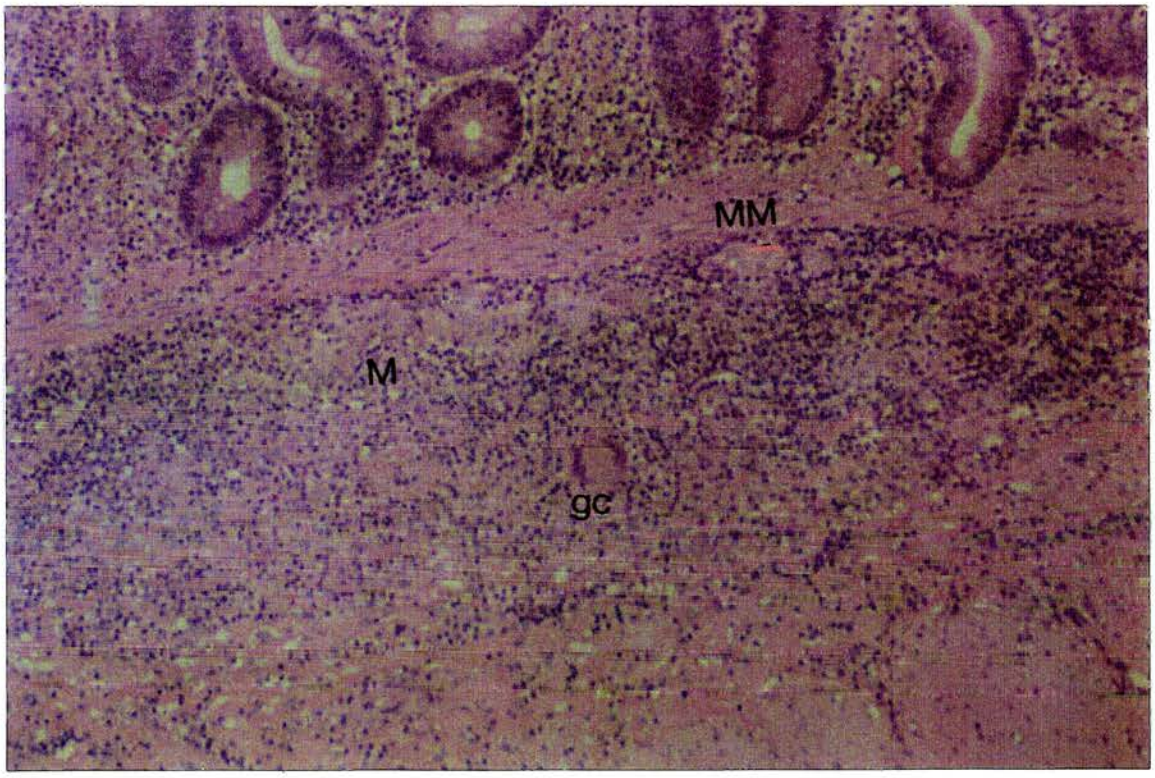


Figure 2.13: Paucibacillary case. Granulomata (g) in the ileal serosa. HE (x100).

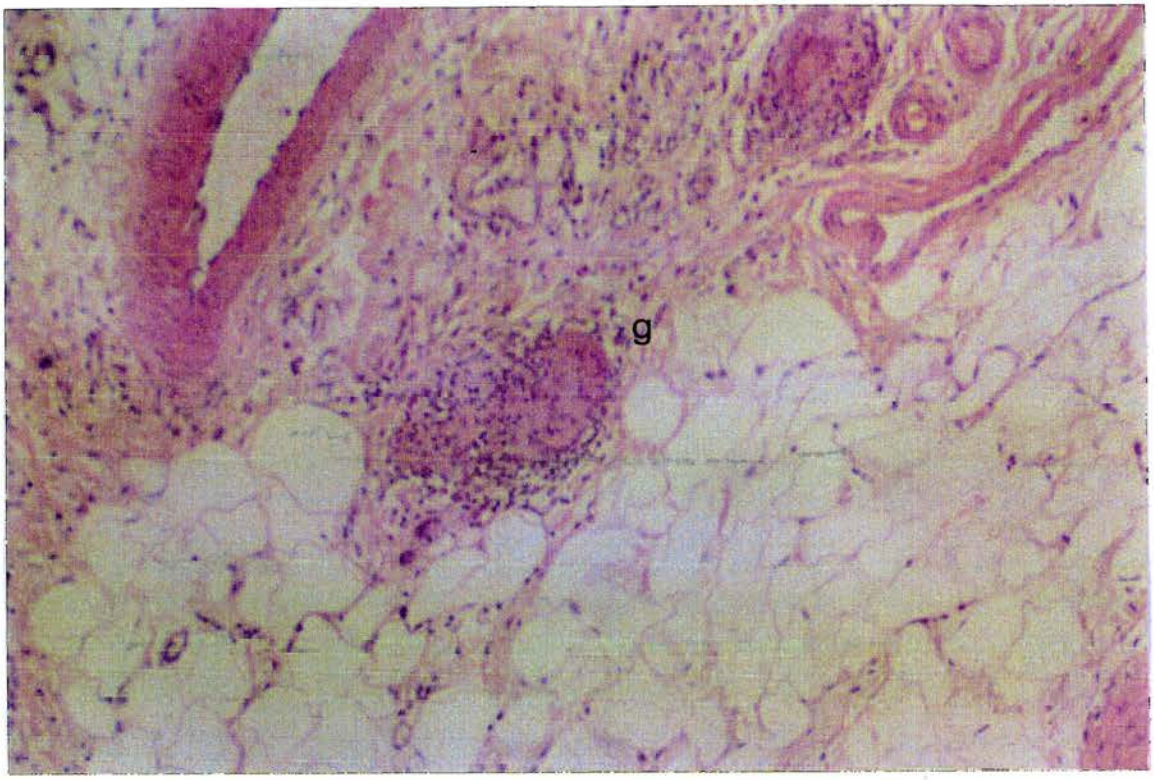
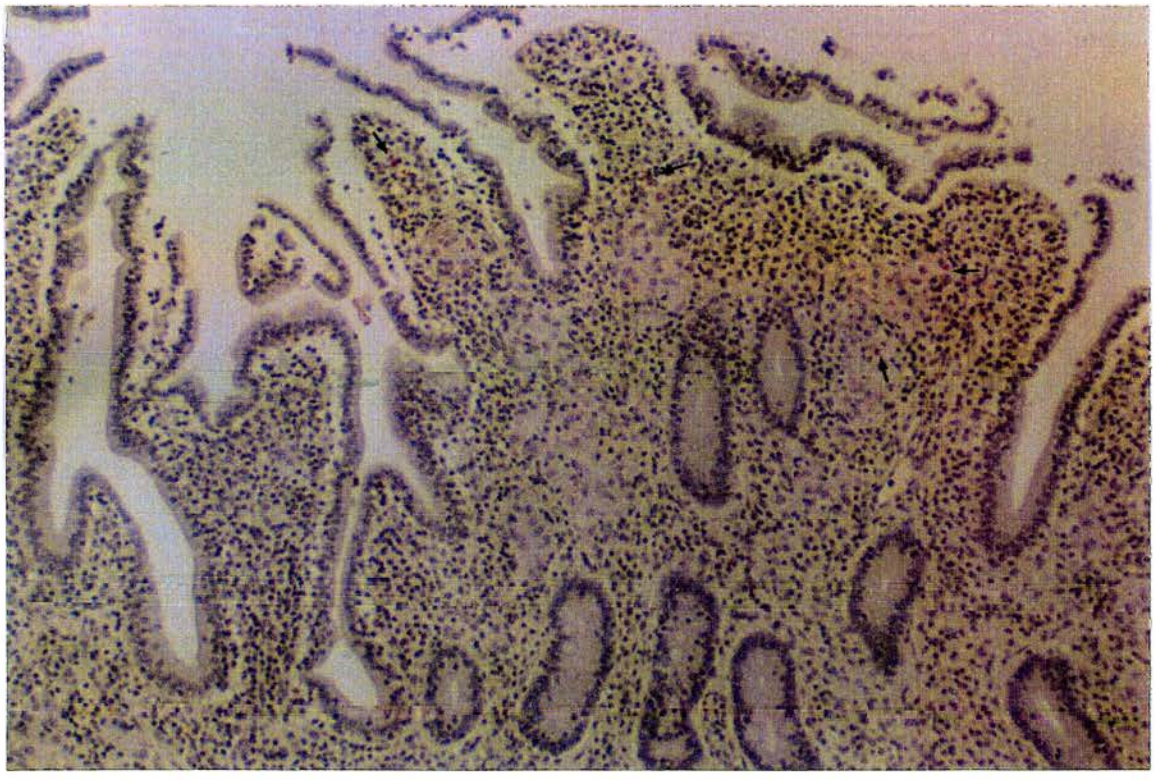


Figure 2.14: Paucibacillary case (P34). Few AFB↑are detectable within granulomatous areas visible in the section. ZN (x100).



observed between the proportion of cells infected with AFB and the bacterial load per cell ($P<0.001$).

An inverse correlation was found between lymphocytic infiltrate and AFB presence, and between lymphocyte infiltration and macrophage infiltration ($P<0.001$).

The presence of giant cells correlated with the presence of a lymphocytic infiltrate ($P<0.02$), and inversely correlated with both bacterial load per cell, and percentage of macrophages infected by AFB (both observations $P<0.01$).

A significant correlation was found between the presence of AFB and a positive serum AGID test result ($P<0.01$).

2.3.10 Morphometric measurements of ileum in diseased and control animals

Mean morphometric measurements in μm are shown in table 2.6. The mean measurements for all observations made on sections of diseased ileum were found to differ significantly from normal, control animals, and would appear to confirm that significant histometric deviations from the normal ileal architecture were present in diseased animals. Full-wall, mucosal, lamina proprial, submucosal, muscular and serosal thickness measurements were all found to be significantly greater in the sections of both multibacillary and paucibacillary cases than in control sections. Villus length was found to be significantly reduced in both groups of diseased animals compared with controls ($P<0.01$ for both groups). In addition, villus flattening and fusion were assessed by scoring on a scale of 0-3 (as for histological and cellular observations), and flattening/fusion found to be significantly greater in both

multibacillary and paucibacillary cases than in control animals (table 2.6). Significant differences between diseased groups were observed in full wall, submucosal, and serosal thickness measurements, which were all found to be significantly higher in the multibacillary group ($P<0.01$). The percentage of glandular tissue in the LP was found to be significantly lower in both diseased groups, but particularly so in the multibacillary group (24.3% compared with 47.4% in normal animals, $P<0.01$).

2.4 DISCUSSION

The results of this study confirm that clinical paratuberculosis in sheep is associated with granulomatous changes of the intestine, in particular the ileum, with a range of gross and histological lesions, which are characterised by two distinct histopathological forms. In the early stages of collection and examination of tissues from diseased sheep, it became apparent that sheep with similar clinical presentation were not consistently found to have the same gross or histological lesions. A useful starting point in the lesional classification proved to be the retrospective division of cases on the basis of AFB burden, and a strong correlation was found between the intracellular bacillary load and the host's immune response, on the basis of the infiltrating cell types and the presence of serum antibody. Positive correlations were observed between AFB load and macrophage infiltrate and a positive serological test, and negative correlations with lymphocytic and giant cell infiltrates.

Despite different lesions and putative differences in the immune responses involved, the clinical presentation of both groups of animals was remarkably similar, the main presenting sign being poor bodily condition. Diarrhoea was present as a clinical sign in 19% of animals of the multibacillary group only, and in fact, in all of these cases appeared to be associated with the presence of lesions in the large intestine, and may have been the result of impaired fluid absorptive capacity. This finding

confirmed the observation that diarrhoea, although an index clinical presenting sign in cattle, is not present in the majority of ovine paratuberculosis cases (Stamp and Watt, 1954; Chiodini *et al.,* 1984), and may be a result of a higher fluid-absorptive capability in this species, or a different degree of involvement of histamine in mediating hypersensitivity reaction as was proposed for bovine paratuberculosis by Buergelt *et al.,* (1978).

The non-specific gross pathological changes of carcase emaciation and oedema were noted in the majority of cases, and more specifically, thickened intestinal wall with luminal dilatation, prominence of serosal lymphatic vessels, and mesenteric lymphadenomegaly were all found. The area of the intestine most consistently affected was found to be the terminal ileum (in keeping with the findings of previous investigators), and indeed this has been proposed as the portal of entry for the organism, perhaps as a result of the high density of GALT at this level (Nisbet *et al.,* 1962; Momotani *et al.,* 1988). Gross changes in the ileum were detectable in 85% of animals, but mucosal ridging, which has been described as a characteristic finding of bovine paratuberculosis, appeared in less than half of the cases, and crevicing of the mucosal surface in yet fewer.

All histological lesions seen during the course of this study correspond with those described by Pérez *et al.,* (1996), as type 3, in particular subtypes 3b (multibacillary) and 3c (paucibacillary), and some with subtype 3a (also paucibacillary). The multibacillary and paucibacillary groups also respectively corresponded with those described as type/group I and type/group II by Stamp and Watt (1954) and by Rajya and Singh (1961), and with the first and second categories (44 and 6 of 50 sheep respectively) by Carrigan and Seaman (1990). In addition, Reddy *et al.,* (1984) described, as type I, lesions comparable with those of the multibacillary group. Pérez *et al.*, (1996), additionally described lesions of types 1 and

2, which were associated in particular with isolated, small granulomata in the ileocaecal Peyer's patch (PP), in the absence of lesions of the intestinal lamina propria and of macroscopic lesions. Although no AFB were detected in either PP tissue or ileal mucosa from a total of forty sheep in that study, almost half gave positive culture results. Those lesions would appear to be early type lesions in the subclinical stages of paratuberculosis, and correspond with the findings in experimentally infected lambs (Nisbet *et al.,* 1962), and would seem to corroborate the hypothesis that ileal PP are portals of entry for the bacteria. (Momotani *et al.,* 1988). Lesions in this study did not correspond since all sheep investigated were presented as clinically affected cases, and in most instances were in the terminal stages of disease. Pérez and co-authors applied pathological studies to all sheep which were culled from individual flocks for any reason, and as a result detection and examination of early stage lesions were possible.

Lesions of caseous necrosis and mineralization have been described in sheep (Stamp and Watt, 1954; Rajya and Singh, 1961; Nisbet *et al.,* 1962), considered to be common and noted to have an appearance similar to lesions of tuberculosis (Barker *et al.*, 1993). However the findings of this study (and in keeping with the findings of Carrigan and Seaman, 1990, and Pérez *et al.,* 1996) did not concur with that observation, with caseous necrosis not being found, and mineralization being largely absent. When evidence of calcification was found in the MLN of three animals, it appeared to be attributable to old, organised parasitic lesions, rather than derived from *Map* lesions, of which no characteristic features were detectable.

The range of lesions described would appear to be considerably more broad than that described in bovine paratuberculosis by Buergelt *et al.,* (1978), who found that cases were characterised by either macrophage or giant-cell infiltrates, with some animals showing a combination of both cell types. However granulomata in varying degrees of organisation were described. The role of lymphocytes as infiltrating cells

was not reported, nor was AFB burden, although it was implied to be lower in cases with predominantly giant-cell infiltrates. In this study, the presence of a macrophage infiltrate has been demonstrated in multibacillary cases, with positive correlations between macrophage number and AFB load, and with the percentage of macrophages infected by AFB. However the presence of Langhans giant cells correlated positively with lymphocyte number, but inversely with AFB number and with the percentage of macrophages having intracellular AFB. In conjunction with the inverse correlation between macrophage and lymphocyte numbers, these observations suggest a different type of host response in each scenario, which although not mutually exclusive are distinctly polarised. The inverse relationship between the presence of high numbers of lymphocytes and the presence of AFB suggests that lymphocytes are active in mediating the clearance of infection.

The two histological forms can thus be categorised in microbiological terms on the basis of the numbers of AFB present (multibacillary or paucibacillary), and each lesional form is associated with distinctive cellular infiltrates of different cell types. The multibacillary form has a marked macrophage infiltrate, whereas the paucibacillary form is associated with an infiltrate of lymphocytes. In mycobacterial infections of humans, in particular leprosy and to a lesser extent tuberculosis, lesions have been categorised on the bases of bacillary number and histological appearance. In leprosy a spectral distribution of lesions has been observed and these have been correlated with the immune response and immune reactions of the individual patients, and a recognised classification developed (Ridley and Jopling, 1966). This spectrum of lesions ranges from organised epithelioid cell granulomata with apparent control over bacillary multiplication (as defined by intracellular AFB burden), and surrounded by a lymphocytic mantle which is tuberculoid leprosy (high immunity 'TT' type), to lepromatous leprosy characterised by sheets of large, mainly unactivated macrophages that contain an abundance of AFB (designated 'LL' type). These two classifications

comprise the polar forms of leprosy, and between both extremes lies a range of lesion types. Midline in this range is borderline leprosy ('BB') which is an intermediate form with less organised epithelioid cell granulomata and higher AFB numbers than the TT polar form. In addition, lesions with a histological character between BB and polar TT or LL forms are graded BT and BL respectively. In leprosy, patients may shift from one lesion type to another, signifying a change in the reactional state or immune status. Patients generally present clinically with the lesions corresponding to BT (with few lesions present), BL or LL positions (with multifocal/widespread lesions) (Lucas, 1988). Patients with TT lesions are often found to self-cure, and lesions are seen to resolve spontaneously. However patients with lesions in the middle of the spectrum tend to progress towards either pole; towards tuberculoid lesions in response to chemotherapy, and towards lepromatous lesions without chemotherapeutic intervention. Patients with simultaneous, significantly different lesion-types present are rare, as are patients with a single LL lesion, giving the impression that control of infection is systemic, and not purely locally controlled (Lucas, 1988).

The similarities between the lesions of human leprosy and ovine paratuberculosis are evident, and comparison between these two diseases has been made previously, with reference both to lesions (nodular/tuberculoid and diffuse/lepromatous) (Pérez *et al.,* 1996; Williams *et al.,* 1983; Chiodini *et al.,* 1984) and to a progression of immune response, initially defined as cell mediated (tuberculoid) but progressing towards humoral (lepromatous) in character (Chiodini *et al.,* 1984). However, the focal granulomata found in paucibacillary cases of paratuberculosis did not appear to be as organised as the characteristic granulomata described in tuberculoid leprosy, which are associated with organised sheets of epithelioid cells (coalescent activated macrophages) and Langhans giant cells, surrounded by a lymphocytic mantle. Paucibacillary lesions were found to be

characterised by dense lymphocytic infiltrate and focal aggregates of macrophages, with few AFB, and not true granulomata in leprosy terms.

The spectrum of lesions in paratuberculosis has been considered to be part of a disease continuum, starting in the early stages with tuberculoid lesions signifying high cell mediated immunity with control over bacillary multiplication and progressing towards the lepromatous form, associated with failure to control bacillary multiplication, an increasingly humoral response and proposed T-cell anergy (Bendixen, 1978; Chiodini *et al.*, 1984). The latter has been defined by the lack of cutaneous DTH response to intradermal *Map* PPD (purified protein derivative) injection in the Johnin skin test (Bendixen, 1978), and sub-optimal PBL proliferation to both concavalin A (ConA) and mycobacterial antigen stimulation (Kreeger and Snider, 1992). This hypothesis may be correct if somewhat simplified, however both forms of lesion are associated with clinical disease and are seen in animals which are highly moribund and in the terminal stages of infection. The immune mechanisms are likely to be more complicated with factors such as cytokine control, antigen presenting cell lineage and costimulatory factors influencing the nature of the immune response towards either a cell mediated/DTH response, or an antibody response. In addition, the role of T cells in influencing the outcome of intracellular infections is likely to be important in the promotion of DTH or antibody type responses. The liberation of large numbers of bacteria from infected macrophages into the extracellular tissues in multibacillary cases may be involved in promoting an antibody type response. The end stage of both DTH and antibody type immunological responses would appear to be disruption of the normal tissue architecture and function resulting in clinical disease.

Mycobacterial virulence, host immune responses and tissues affected may all play a part in determining the histological appearance of lesions associated with mycobacterial disease. *M. tuberculosis* and *M. bovis* infections appear to be associated

with higher degrees of tissue necrosis than do *M. leprae* or *M. a. paratuberculosis* infections, which demonstrate lower cytotoxicity to the host cell. Disease in the latter two infections is associated with hypersensitivity and granulomatous reactions to mycobacterial antigens. The granulomata present in both of the former diseases exhibit a spectral nature also, in respect to their histological classification. (Lucas, 1988). In tuberculosis, granulomata can be non-necrotic, epithelioid cell granulomata, through granulomata with central caseation necrosis and higher AFB burdens, to liquefactive, necrotic granulomata with fibrosis and abundant extracellular AFB in the coagulative centre. The lesions may be a response to inherent mycobacterial cytotoxicity, or virulence factors such as the ability to induce necrosis, or a rapid growth rate in comparison with *M. leprae*. Likewise in paratuberculosis different mycobacterial strains may possibly be associated with differential virulence. It has been noted in this study that multibacillary lesions were associated with deposition of pigment in the mucosa, but this was not found in any of the paucibacillary forms. It could be hypothesised that pigmented strains either fail to induce a strongly cell mediated immune response, or tend to induce lepromatous type lesions, either in correlation with AFB number (for example as a result of fast growth rate) or by direct influence on the host response. Alternatively, pigment may be visible in these cases purely as a result of high numbers of AFB, although the lesions of pigmented and non-pigmented multibacillary cases showed no variation. Different forms of mycobacterial LAM have been shown to induce differential responses in macrophages, either by means of their ability to stimulate a pathway of TNFα production and thereby improving the efficacy of the host macrophage's response, or by the ability to evade host-macrophage responses by interference with the respiratory burst (Kaufmann, 1993).

The tissue in which the lesions are found may also dictate to some extent the lesion morphology, with gut lesions likely to have a different appearance from granulomata in cutaneous tissues.

The host immune response would appear to play a definitive role in relation to the type of lesions seen, and is the subject of subsequent chapters.

In the paucibacillary group, the absence or scarcity of AFB resulted in concern that the lesions were not in fact a result of *Map* infection. In order to confirm *Map* infection, genomic DNA was extracted from ileal samples and subjected to PCR for *Map* DNA. IS900 DNA sequences, specific for *Map*, were detected in all of the ileal tissue samples tested from sheep with multibacillary lesions, and in nine out of the fourteen animals with paucibacillary lesions (Chapter 8). Two of the remaining five cases had detectable AFB in the ileum and in MLN, and a further two had demonstrable serum antibody as detected by the AGID test. In the fifth animal, a strong positive IFNγ response was detected by ELISA (Rothel *et al.,* 1990) when PBL were stimulated with *Map* antigen (procedure described in Chapter 7). Condron *et al.*, (1994) reported the presence in paratuberculosis granulomata of spheroplast forms of *Map* in which the bacterial cell wall was absent, but the cell membrane intact. As such the organisms were not visible by conventional ZN staining and light microscopy, but were detectable by electron microscopy. In addition, Plante *et al.*, (1996) reported bovine paratuberculosis cases in which no AFB were seen on ZN staining, but in which IS900 sequences were detectable by PCR technique.

Morphometric measurements of histological parameters showed significant change in the ileal architecture of both groups of diseased animals compared with normal controls. Significant thickening of the ileal wall, mucosa, lamina propria, submucosa, and serosa were present in the diseased groups compared with controls, while villus length was found to be significantly decreased in diseased animals. Submucosal, serosal, and full wall thickness were greater in the multibacillary than the paucibacillary group. It would appear that, irrespective of the differences in cellular populations and lesions, the resultant change in the ileal morphology was remarkably

similar in diseased animals of both groups, and so may account for the lack of variation in the clinical presentation of all cases.

CHAPTER THREE

CHARACTERISATION OF THE INTESTINAL LYMPHOCYTE BY IMMUNOHISTOCHEMISTRY

3.1 INTRODUCTION

The immune cell populations of normal sheep and in the lesions of paratuberculosis have not been extensively investigated. Most immunological studies in paratuberculosis have been performed in the bovine, in particular with reference to peripheral blood lymphocytes and their responses to mycobacterial antigens (Kreeger and Snider, 1992; Kreeger *et al.*, 1992; Chiodini and Davis, 1992; Chiodini and Davis, 1993). This study is concerned with the lymphocytes of the distal ileum, which is the consistent site of infection and lesions in the sheep. The aim of this study was to characterise phenotypically the lymphocyte subsets present in normal sheep ileum, and to discern any difference in this population in paratuberculosis affected sheep which may implicate their involvement in the responses to *Map* infection. In any infection, the outcome is affected by the immune response of the host, and the pathogenic mechanisms of the pathogen itself, and in the case of persistent infections, a balance between these two factors. In paratuberculosis, the persistence of the infected macrophage suggests an apparent failure of the host's local enteric immune response to eliminate the pathogen by cell-mediated immune mechanisms normally employed against the mycobacteria, and intracellular pathogens in general. Intracellular pathogens, which include the mycobacteria, are capable of evading many of the host's defences, especially humoral mechanisms which are effected by antibodies. Antibodies mediate clearance of extracellular pathogens in a number of different but interconnected ways, either by neutralisation, opsonisation allowing phagocytosis, antibody dependant cytotoxicity, or by triggering complement cascade mechanisms.

Immunity to intracellular infections, however, is dependant on cell mediated mechanisms, which ultimately result in elimination of the pathogen by intracellular killing by infected macrophages. Lymphocytes are principal effectors in cell mediated immunity. Recruitment of lymphocytes to sites of infection and proliferation of certain lymphocyte functional and phenotypical subsets is essential in the amplification of the effector mechanisms against intracellular pathogens. Lymphocytes of the $CD4^+$ phenotype are helper/inducer cells which provide cytokine help, resulting in upregulated intracellular killing by macrophages, attraction of further lymphocytes into lesions, promotion of granuloma formation, stimulation of cytotoxic T cells and NK cells, and the promotion of inflammatory responses. T cells of $CD8^+$ phenotype are principally cytotoxic in action, and can also produce an array of cytokines in the manner of T-helper cells. A suppressor role for $CD8^+$ lymphocytes has been described, classically in lepromatous leprosy lesions, and in the promotion of tolerance to ingested antigens, and self antigens. Cytokines are of primary importance in the immune responses to mycobacteria (reviewed by Moreno and Rees, 1993). Mycobacteria-infected macrophages produce proinflammatory cytokines including TNFα, IL-6, IL-1α and β, and GM-CSF in response to mycobacterial components such as LAM and peptidoglycan, and present antigen to $CD4^+$ cells in association with MHCII. T helper cells are capable of producing a range of cytokines in response, either for promotion of an antibody response, or help for macrophages. IFNγ has been found to be a potent macrophage stimulator which can upregulate intracellular killing by these cells, and TNFα also appears to play an important stimulatory role in facilitating clearance of intracellular organisms (Flesch and Kaufmann, 1990). Lymphocyte phenotype has been linked to important immune functions in a variety of mycobacterial infections. In leprosy, predominance of certain T-cell subsets has been implicated in correlation with histopathological forms of the disease (Van Voorhis *et al.*, 1982). $CD4^+$ cells have been shown to be the predominant cell T-cell subset in the infiltrates of cutaneous tuberculoid lesions, and $CD8^+$ cells are associated with

lepromatous lesions. In tuberculous pleurisy, $CD4^+$ cells have been found to be the predominant cell type (Barnes *et al.,* 1989). In the mouse, $CD4^+$ cells have been categorised on the basis of function into Th1 and Th2 subpopulations (Mosmann *et al.*, 1986). In an effective immune response, $CD4^+$ helper T-cells of Th1 type produce IFNγ which promotes intracellular killing by infected macrophages (Nathan *et al.*, 1983). A Th2 type of response resulting in downregulation of Th1 type responses and the promotion of antibody production would appear to be inappropriate in a mycobacterial infection. $CD8^+$ cells are necessary for a protective immune response to *M. tuberculosis* infection (Flynn *et al.*, 1992), and are present in large numbers in lepromatous lesions of leprosy in which a suppressor role has been described for this subset (Modlin *et al.*, 1986). Gamma-delta T cells accumulate at the site of mycobacterial infections (Janis *et al.*, 1989; Modlin *et al.*, 1989) and are considered to play a role in the regulation of granuloma formation (Mombaerts *et al.*, 1993). This subset is particularly prominent in ruminants (Hein and Mackay, 1991).

Gorrell *et al.* (1988), examined lymphocyte phenotypes in the duodenum and jejunum of foetal sheep and of lambs of 5-9 months using immunohistochemistry. Studies on humans, mice, rats, pigs and calves have shown distinctive distribution patterns of T-cell subsets in the intestinal mucosa (Selby *et al.*, 1981; Janossy *et al.*, 1980; Vega-López *et al.*, 1993; Parsons *et al.*, 1993).

It was decided in the first instance to employ immunohistochemical staining in order to determine the distribution and density of positively stained cells and appreciate the cell and tissue morphology. Immunochemical staining of tissue sections provides a method whereby the density and location of postively stained cells can be determined in addition to percentages of positively stained cells present. Flow cytometry alone does not allow enumeration of density of cells in the tissue or their distribution *in situ.* The use of monoclonal antibodies in conjunction with the avidin/biotin/peroxidase

complex method (Hsu *et al.*, 1981) was chosen for this purpose. Investigation of the T-lymphocyte subsets present in normal ileum and any alteration or imbalance in this population in the lesions of paratuberculosis may provide information on the persistence of infection. This study attempted to characterise phenotypically the T-cell subsets of the ileum *in-situ* by immunohistochemistry. The proportions and distribution of intestinal lymphocyte subsets were examined both in normal, non-infected sheep, and in sheep with clinical paratuberculosis .

3.2 MATERIALS & METHODS

3.2.1 Animals

Thirty adult sheep of mainly Scottish Blackface and Cheviot breeds, comprising 18 adult naturally paratuberculosis infected, clinically diseased sheep and 12 healthy adult sheep were euthenised by intravenous pentabarbitone injection. Sheep were given a clinical examination *ante mortem*, and serologically tested for *Map*-specific antibodies using the AGID test (Sherman *et al.*, 1984). Control sheep were confirmed as being non-diseased by serology, clinical examination, necropsy and histology. Paratuberculosis infected sheep were classified according to histological lesions into lepromatous (n=12) and tuberculoid (n=6) groups as described in chapter 2. Table 3.1 contains the identifying numbers of the sheep used in this study, and their breeds and ages.

3.2.2 Samples

Full necropsy examinations were carried out during which fresh samples of distal ileum and MLN were collected and snap frozen in solid CO_2/ isopentane slush, orientated on cork discs, immersed in 'Optimum Cutting Temperature (OCT) Compound' cryoprotectant embedding medium (Miles, Elkhart, IL) and stored at -70°C until required. Gut and MLN samples were also taken and fixed in buffered formalin solution and processed routinely for histological examination. Sections were cut and stained with haematoxylin and eosin, and by the Ziehl-Neelsen method for the detection of acid-fast organisms (section 2.2.4).

3.2.3 Monoclonal antibodies

The monoclonal antibodies that were employed in the immunohistochemical staining, and their specificities, are listed in table 3.2. Saturated hybridoma supernatants were used neat, but ascitic fluid MAbs were titrated, and the dilutions are specified in the table. Monoclonal antibodies used in quantified staining were 'SBUT4' and 'SBUT8' against ovine CD4 and CD8 leukocyte antigens respectively (Maddox *et al.*, 1985), and '86D' against ovine γδ T-cell receptor (δ chain specific) (Mackay *et al.*, 1989).

Table 3.1: Details of sheep included in this study.

Control			*Lepromatous*			*Tuberculoid*		
Sheep	*Breed*	*Age*	*Sheep*	*Breed*	*Age*	*Sheep*	*Breed*	*Age*
1	Blf x Tex	18m	P2	Texel x	18m	P8	Blackface	Adult
2	Blf x Tex	18m	P3	Blackface	Adult	P19	Greyface	Adult
3	Blf x Tex	18m	P4	Blackface	Adult	P23	Cheviot	Adult
4	Blf x Tex	18m	P5	Blackface	Adult	P25	Blackface	Adult
5	Blf x Tex	18m	P6	Blackface	Adult	P27	Cheviot	Adult
6	Blf x Tex	18m	P9	Blackface	Adult	PC39	Blackface	Adult
C3	Blackface	Adult	P10	Blackface	Adult			
C5	Blackface	18m	P11	Greyface	Adult			
C7	Blackface	Adult	P13	Blackface	Adult			
C24	Blackface	Adult	P14	Cheviot	2yr			
C25	Greyface	Adult	P15	Blackface	Adult			
C29	Blackface	Adult	P16	Cheviot	Adult			

Blf: Blackface; Tex: Texel

Table 3.2: Monoclonal antibodies and their specificities. SS: saturated hybridoma tissue culture supernatant

Antibody	*Specificity*	*Dilution*	*Reference*
SBUT4	Ovine CD4	1:1000	Maddox *et al.*,1985
SBUT8	Ovine CD8	1:1000	Maddox *et al.*,1985
86D	Ovine γδ TCR	SS	Mackay *et al.*,1989
CC15	Bovine T19	SS	Clevers *et al.*,1990
DU2,87	Ovine B cell CR2	SS	Hein *et al.*,1995
VPM65	Ovine CD14	SS	Gupta, 1994
VPM36	Ovine MHCII DQα	SS	Dutia *et al.*,1993

3.2.4 Immunoperoxidase staining technique

Sections were stained using the 'VectorStain Elite ABC Kit' (Vector Labs. Peterborough, UK.). All reagents were supplied in this kit with the exception of PBS, primary monoclonal antibodies (table 3.2), and normal mouse serum.

Serial sections of frozen tissue were cut at 6-8 microns on a cryotome onto glass slides which had been pre-treated with Vectabond tissue section adhesive (Vector) and allowed to air dry at room temperature for two hours. Sections were fixed in cold acetone for 5 minutes at 4°C and allowed to air dry for 5 minutes at room temperature before being mounted in coverplates (Shandon, Basingstoke, UK.), which were then placed into a Shandon 'Sequenza' apparatus and washed three times in PBS, pH 7.4. The Shandon coverplate system allowed the section to be kept in a moist environment while preventing evaporation of reagents, each coverplate holding approximately 90 µl of liquid at all times, and facilitated thorough washing by gravitational flow. Three washes through the coverplate was equivalent to approximately 10 minutes of washing.

Endogenous peroxidase activity in the tissue was then blocked using a glucose oxidase procedure (appendix 3.1) based on that described by Andrew and Jasani, (1987), in which nascent hydrogen peroxide is elaborated. This procedure was effective in blocking endogenous peroxidase while maintaining tissue morphology. Initial attempts to block endogenous gut peroxidases were made using conventional hydrogen peroxide and methanol/hydrogen peroxide methods, but these resulted in tissue damage and section disintegration. The glucose oxidase system was tested at various stages in the immunostaining protocol and for different incubation times. An incubation period of one hour, after initial washing and before the application of other

reagents, was found to be optimal in quenching peroxidase activity while maintaining tissue architecture. Sections were incubated in 100 µl glucose oxidase solution for 1 hour at 37°C. The sections were then again washed thoroughly to remove all traces of glucose oxidase/hydrogen peroxide by three washes in PBS. Non-specific binding of the monoclonal antibodies was blocked by the addition of 100 µl of 1.5% normal horse serum (diluted in PBS) and incubation for 15 minutes at room temperature. Primary monoclonal antibodies were then added to the sections (100 µl) which were then incubated overnight (approximately 16 hours) at 4°C. Negative control slides were included by omission of primary antibody, and by using normal mouse serum diluted 1:100 in PBS in place of primary antibody. The three step PBS wash process was repeated and 100 µl biotinylated secondary antibody (horse anti-mouse) was added and incubated at room temperature for 30 minutes. Three washes of PBS (10 minutes) followed before the addition of 100 µl of pre-formed '**a**vidin and **b**iotinylated horseradish peroxidase macromolecular **c**omplex' (ABC) solution. This was incubated at room temperature for a further 30 minutes, and then washed off by three PBS washes as described.

Positive staining was visualised using the chromogen diaminobenzidine (DAB) (Vector). Sections were incubated in DAB solution for 6 minutes and the visual product darkened by the addition of nickel chloride solution (which was included in the DAB kit) to the reaction. The slides were removed from the coverplates at this point and washed in a large volume of tap water, counterstained in Meyer's haematoxylin for 1 minute, washed again in tap water, immersed in Scott's tap water substitute for 1 minute and finally rinsed in tap water. The sections were dehydrated through graded ethanol and cleared in xylene, and mounted in DPX mountant (BDH, Poole, Dorset) using glass coverslips.

3.2.5 Enumeration of positively stained cells

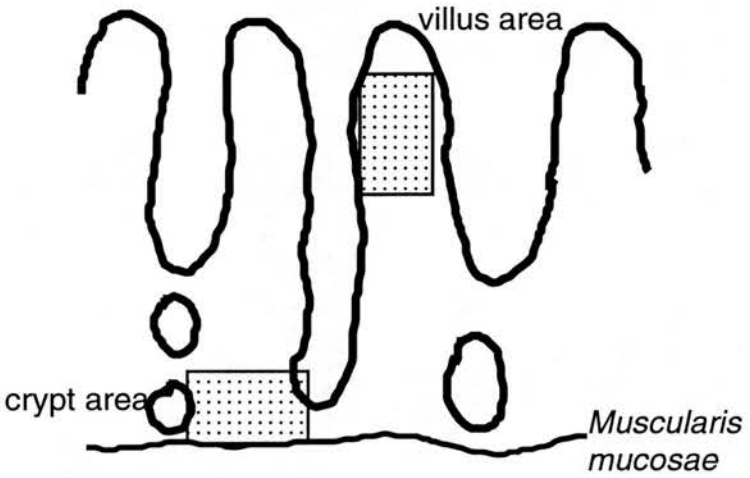
Positively stained cells were counted through a microscope at 200x magnification using a rectangular graticule which measured a field of 0.32 x 0.21 mm (0.0672 mm^2). For each tissue section, four graticule fields were chosen at random from villus areas and four from crypt areas. The long edge of the rectangle was aligned along the villus epithelium, and in the crypt areas along the *muscularis mucosae* (Figure 3.1). Within these fields, cells were counted and categorised as being lamina propria (LPL), or epithelial-associated lymphocytes (EAL). EAL were classified as being within the intestinal epithelium or around the epithelial basement membrane because it became evident on preliminary observations that cells could not be described in terms of intraepithelial and lamina proprial cells only. For the purposes of subjective classification, positively stained cells within a depth of two nuclei of the epithelial cell base were considered to be EAL. A better approach would have involved the histochemical staining of the epithelial basement membrane for the purposes of demarcation.

Intraepithelial lymphocytes (IEL) were quantified by counting the positively stained cells in ten lengths of villus epithelium, each measuring 0.32 mm, for each stained tissue section. Cells were defined as being IEL if they were enclosed within the epithelium with the nucleus being at the same level as, or apical in relation to, the epithelial-cell nuclei.

3.2.6 Statistics

'Minitab For Windows' (Minitab Inc., State College, PA.) was used for the statistical analysis of the data. Immunostaining cell-count data were found to be non-

Figure 3.1: Diagram of the areas chosen for counting of positively stained cells. Five fields from villus and five from crypt areas were chosen. The edge of the graticule field was aligned with the villus epithelium and cells counted as LPL or EAL. For crypt areas, the edge of the graticule field was aligned with the *Muscularis mucosae.*



normally distributed by means of normal plots, and were therefore analysed using non-parametric tests. One-way analysis of variance was initially assessed by the Kruskall-Wallis test, and then differences between two groups by the Mann-Whitney test. Statistical significance was taken to be $P<0.05$.

3.3 RESULTS

Preliminary observations suggested that B cell, macrophage and MHC class II staining was not of a discrete enough nature to be readily quantifiable by immunohistochemistry. Consequently, only T-cell subsets were subjected to enumeration. Detailed cell counts are included in appendices 3.3-3.4. MLN staining did not reveal any apparent differences and was more difficult to enumerate satisfactorily using cell counting technique. MLN were frequently the secondary site of lesions (chapter 2) but in spite of this on preliminary observation no differences were notable in the immunohistochemical staining patterns and consequently flow cytometry was chosen to examine MLNL populations (chapter 4).

3.3.1 Distribution of T-lymphocyte subsets within the ileal mucosa

For all three groups of animals, non-infected, lepromatous and tuberculoid, qualitative and quantitative observations showed that each T-cell subset differed significantly in distribution throughout the mucosa (table 3.3; figures 3.2-10).

Higher densities of all three lymphocyte subsets were present in villus than in crypt areas, both in infected and non-infected animals ($P<0.01$). $CD4^+$ cells were situated in the central lamina propria area of the villus around the central lacteal vessel. Very few $CD4^+$ cells were found within the villus and crypt epithelium ($P<0.01$) in

Table 3.3: Distribution of T-cell subsets between mucosal compartments: villus and crypt areas (median and range). Significant differences between compartments are denoted ** (P<0.01) and * (P<0.05).

Group	*Subset*	*Villus total*	*Crypt total*
Control	$CD4^+$	275.5(83-428)	77.5(42-124)**
	$CD8^+$	372(182-726)	89.5(63-155)**
	$\gamma\delta$ TCR^+	62.5(43-139)	36(18-52)**
Lepromatous	$CD4^+$	180(93-263)	69.5(32-142)**
	$CD8^+$	225(79-356)	80(38-235)**
	$\gamma\delta$ TCR^+	66.5(34-118)	31(12-78)**
Tuberculoid	$CD4^+$	423(361-626)	264(105-340)**
	$CD8^+$	476.5(301-656)	148(116-251)**
	$\gamma\delta$ TCR^+	166.5(95-220)	67(39-98)*

Table 3.4: Total cell densities for lamina propria and epithelial associated lymphocytes (median and range). Significant differences between compartments are denoted ** (P<0.01) and * (P<0.05)..

Group	*Subset*	*LP total*	*EA total*
Control	$CD4^+$	342.5(167-498)	1(0-10)**
	$CD8^+$	245(74-581)	241(145-465)
	$\gamma\delta$ TCR^+	55(23-147)	41.5(18-76)*
Lepromatous	$CD4^+$	247.5(123-349)	3(0-18)**
	$CD8^+$	143.5(79-255)	153(53-277)
	$\gamma\delta$ TCR^+	63(30-116)	27.5(10-62)**
Tuberculoid	$CD4^+$	688(492-879)	2(0-10)**
	$CD8^+$	343.5(274-461)	222(109-507)
	$\gamma\delta$ TCR^+	120.5(85-159)	108(22-215)

both infected and non-infected animals (figure 3.2). $CD8^+$ cells by contrast were located predominantly in close association with the epithelium around the basement membrane, and superficially situated in the lamina propria. $CD8^+$ cells were also present as IEL (figure 3.3). Although the epithelial-associated distribution was immediately apparent, $CD8^+$ cells were also distributed throughout the lamina propria. Gamma-delta TCR^+ lymphocytes were fewer in number than $CD4^+$ and $CD8^+$ subsets and were more widely and sporadically distributed throughout the mucosa, both throughout the lamina propria and within and around the epithelium (figure 3.4).

The cell counts, ratios and relative percentages of cells for normal, control sheep are included in tables 3.3-3.8 from which the distribution of cells between the compartments is evident.

The distribution of lymphocytes around focal granulomatous lesions was assessed subjectively, but preliminary observations indicated no alterations in the pattern of distribution of cell subsets associated with focal granulomas (figure 3.11).

3.3.2 Densities of lymphocytes

Tuberculoid cases were found to have significantly higher densites of $CD4^+$ lymphocytes in the gut than control animals ($P<0.001$) and lepromatous cases ($P<0.001$). Similarly, these cases had higher densities of $\gamma\delta$ TCR^+ cells than control animals ($P<0.05$) and lepromatous cases ($P<0.05$). The $CD8^+$ density was significantly higher than lepromatous cases only ($P<0.01$). Lower densities of both $CD4^+$ and $CD8^+$ lymphocytes were found in lepromatous tissues compared with control ileum ($P<0.01$). However, no significant difference was found in the densities of $\gamma\delta$ TCR^+ lymphocytes between lepromatous and control ileum ($P=0.74$). These

Table 3.5: Total cells counted for each mucosal compartment (median and range). Significant differences from the control group are denoted *** (P<0.001), ** (P<0.01), and * (P<0.05). Significant differences of tuberculoid from lepromatous group are denoted ### (P<0.001), ## (P<0.01), and # (P<0.05)

Observation	*Subset*	*Control*	*Lepromatous*	*Tuberculoid*
Total cells	$CD4^+$	343(167-503)	250.5(125-352)**	688.5(502-880)***###
	$CD8^+$	458(255-881)	291.5(132-532)**	641.5(418-815)##
	$\gamma\delta TCR^+$	98.5(67-187)	93(58-174)	230(134-314)*#
LPL total	$CD4^+$	342.5(167-498)	247.5(123-349)**	688(492-879)**###
	$CD8^+$	245(74-581)	143.5(79-255)*	343.5(274-461)###
	$\gamma\delta TCR^+$	55(23-147)	63(30-116)	120.5(85-159)**##
EAL total	$CD4^+$	1(0-10)	3(0-18)	2(0-10)
	$CD8^+$	241(145-465)	153(53-277)**	222(109-507)
	$\gamma\delta TCR^+$	41.5(18-76)	27.5(10-62)	108(22-215)
Villus total	$CD4^+$	275.5(83-428)	180(93-263)***	423(361-626)***###
	$CD8^+$	372(182-726)	225(79-356)**	476.5(301-656)##
	$\gamma\delta TCR^+$	62.5(43-139)	66.5(34-118)	166.5(95-220)*#
Crypt total	$CD4^+$	77.5(42-124)	69.5(32-142)	264(105-340)**##
	$CD8^+$	89.5(63-155)	80(38-235)	148(116-251)*#
	$\gamma\delta TCR^+$	36(18-52)	31(12-78)	67(39-98)*##

Figure 3.2a: Normal sheep (sheep C29). Photomicrograph of representative field of ileal mucosa. Immunoperoxidase staining of CD4 (SBUT4) positive cells. $CD4^+$ cells are present in the central villus lamina propria. (x100).

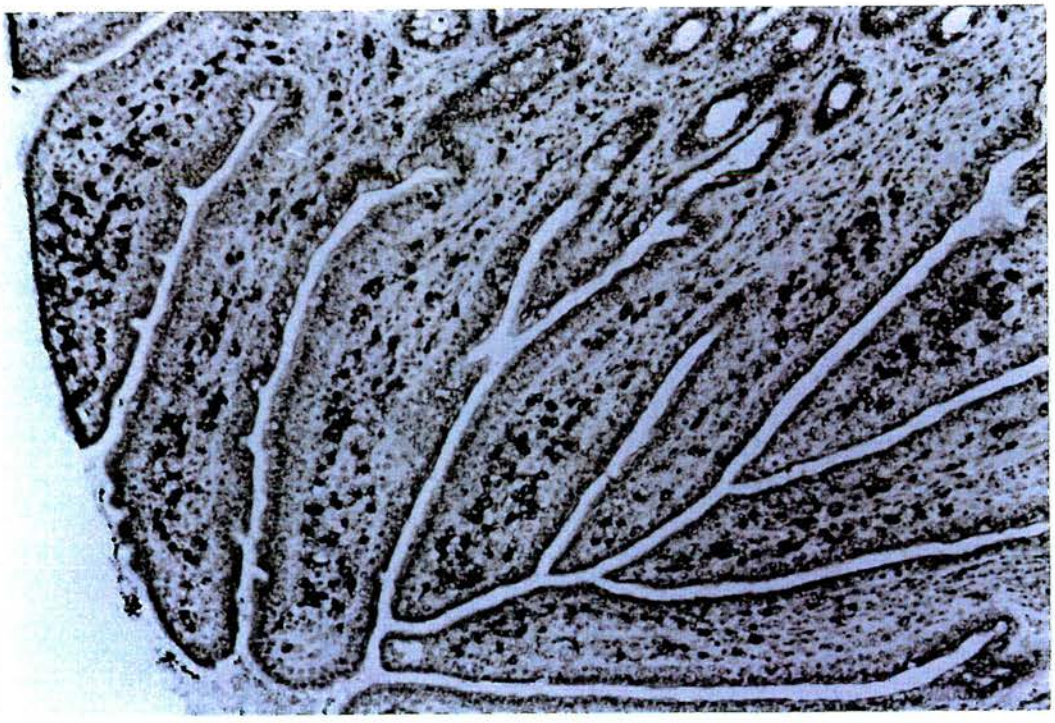


Figure 3.2b: Normal sheep (sheep 2). Higher power photomicrograph of $CD4^+$ cells in normal ileal villi. Immunoperoxidase (x200).

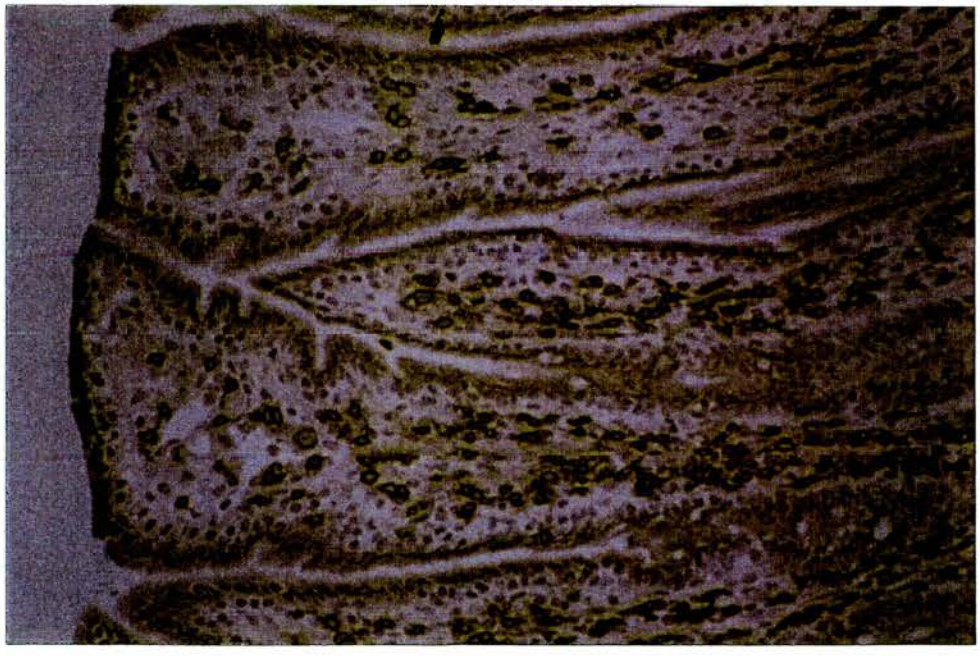


Figure 3.3a: Normal sheep (sheep C29). Photomicrograph of representative field of ileal mucosa. Immunoperoxidase staining of $CD8^+$ (SBUT8) cells reveals high density of positively stained cells in close association with intestinal epithelium. (x100).

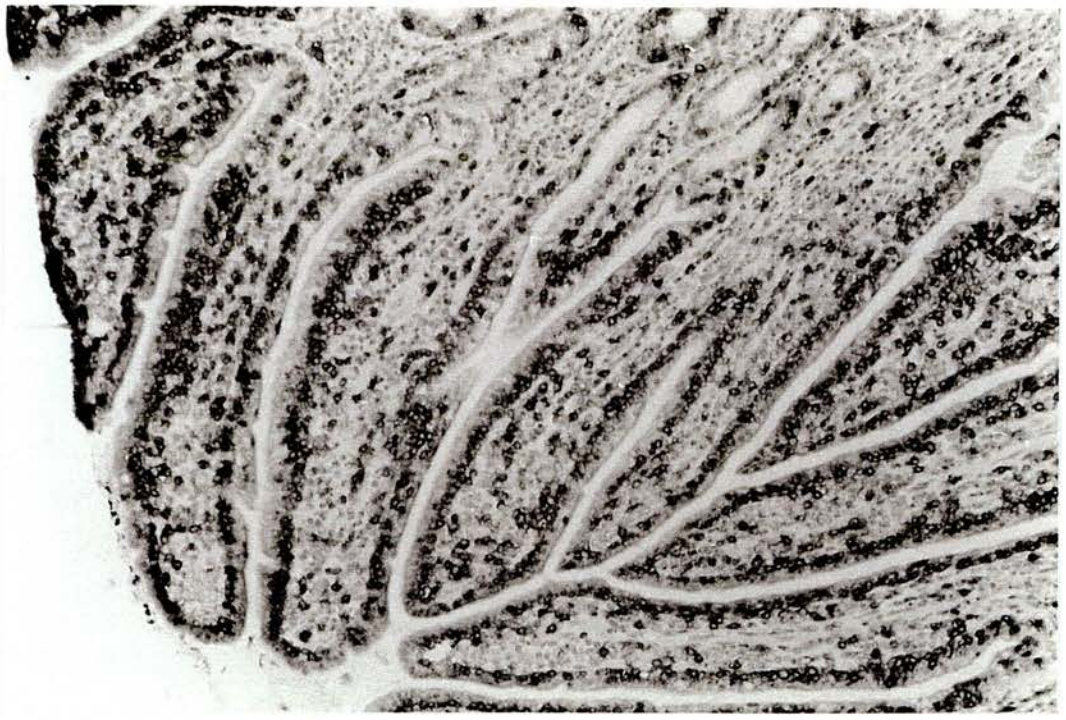


Figure 3.3b: Normal sheep (sheep 2). Higher power photomicrograph of $CD8^+$ cells in the villi of normal ileum. Arrow denotes intraepithelial lymphocyte. Immunoperoxidase (x200).

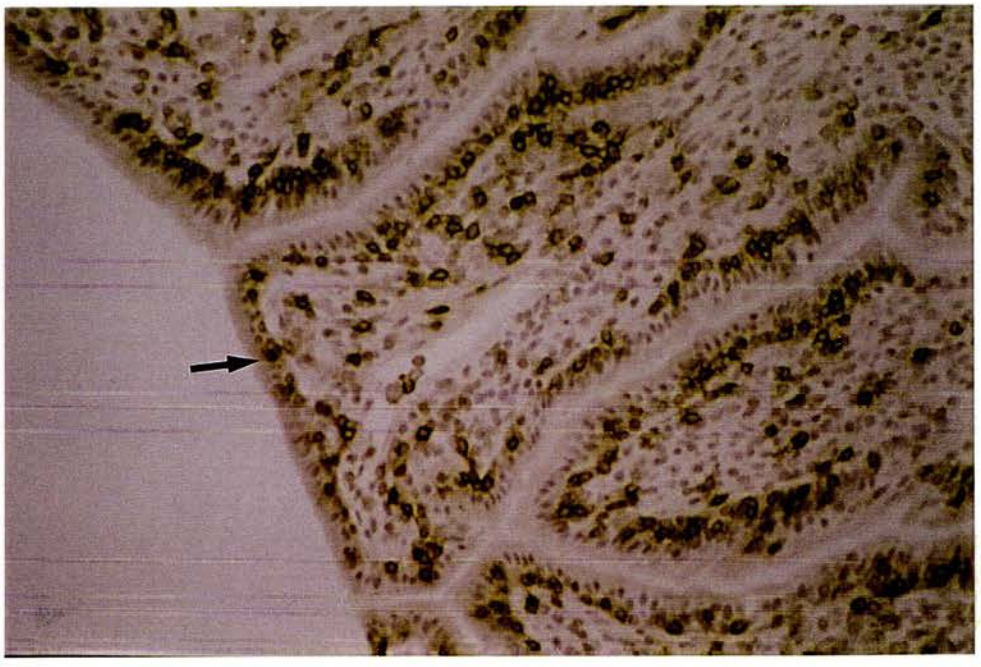


Figure 3.4a: Normal sheep (sheep C29) Photomicrograph of representative field of ileal mucosa. Immunoperoxidase staining of γδ TCR (86D) positive cells reveals both lower density and scattered distribution for this subset compared with $CD4^+$ and $CD8^+$ cells (figures 3.2 & 3.3). (x100).

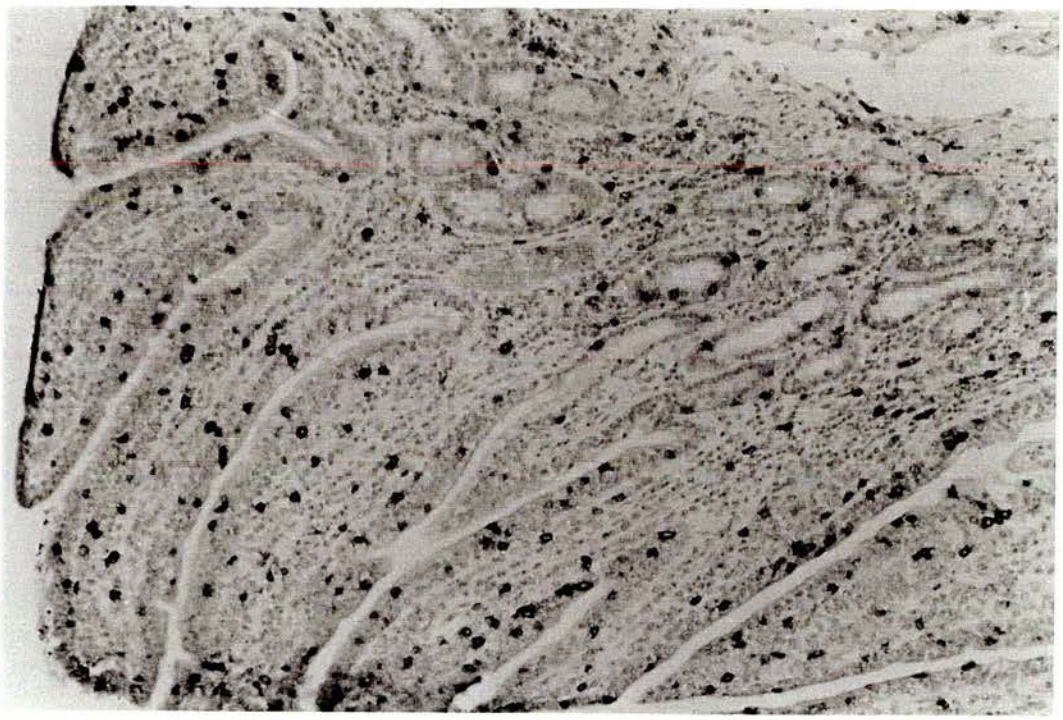
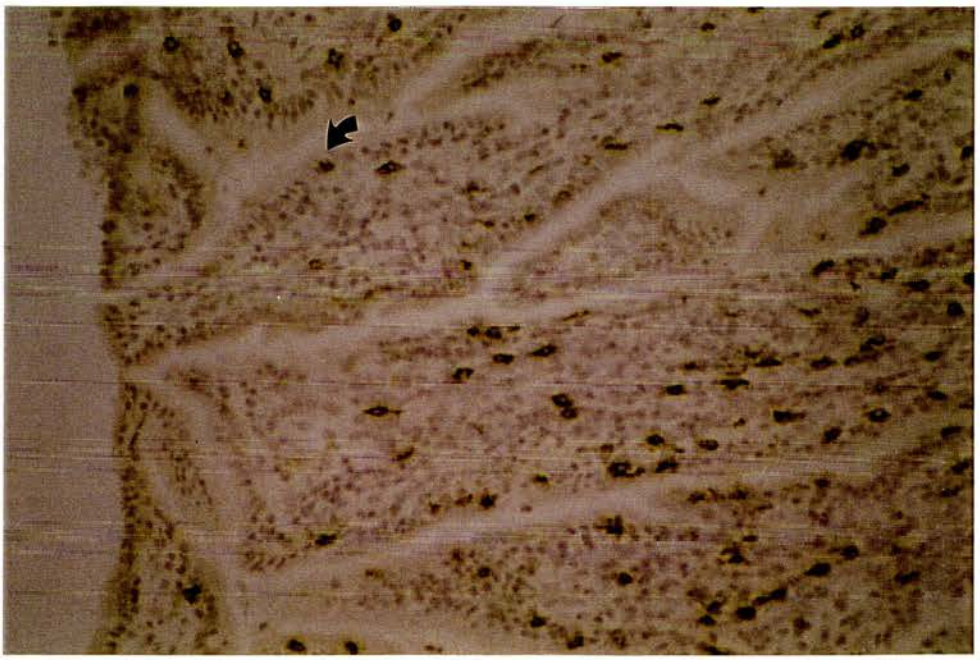


Figure 3.4b: Normal sheep (sheep 2). Higher power photomicrograph of γδ TCR^+ cells in villi of normal ileum. Arrow denotes intraepithelial lymphocyte. Immunoperoxidase (x200).



observations were true for total ileal cells, total villus cells, total villus lamina propria cell compartments (significance levels shown in table 3.5). For crypt areas, the densities of all three subsets were significantly higher in tuberculoid than both normal animals and lepromatous cases, however there were no significant differences between lepromatous cases and normal animals. This suggests that the crypt areas are more affected by the tuberculoid form of the disease than the lepromatous form.

3.3.3 Relative percentages of T-cell subsets

The relative percentage of each cell subset was calculated by expressing the number of cells counted for a particular subset as a percentage of the sum of all cells counted for all three subsets (table 3.6).

Relative percentages of the total cells counted for both the $CD4^+$ and $CD8^+$ subsets for lepromatous versus non-infected animals were not significantly different. However, the relative percentage of the $\gamma\delta$ TCR^+ subset was significantly greater in the lepromatous group than in the non-infected animals ($P<0.05$). In the tuberculoid group, the relative percentage of $CD4^+$ cells was significantly increased ($P<0.05$), and that of $CD8^+$ cells correspondingly decreased ($P<0.01$) compared with non-infected animals.

For total LPL counted, there was an increase in the relative percentage of $\gamma\delta$ TCR^+ cells in the lamina propria of lepromatous compared with control and tuberculoid cases ($P<0.01$). The villus lamina propria population differed in that tuberculoid cases had a significantly increased relative percentage of $CD8^+$ cells compared with lepromatous and control animals ($P<0.05$), and a decreased relative percentage of $CD8^+$ cells in lepromatous compared with control villus lamina propria

Table 3.6: Relative percentages of T-cell subsets (median and range). Significant differences from the control group are denoted *** (P<0.001), ** (P<0.01), and * (P<0.05), and differences of tuberculoid from lepromatous group are denoted ## (P<0.01), and # (P<0.05).

Compartment	*Subset*	*Control*	*Lepromatous*	*Tuberculoid*
LP-villus	CD4+	54.2(40.7-65.9)	51.3(37.3-72.9)	58.4(53.9-61.5)
	CD8+	37.4(29.5-53.2)	18.1(7.1-54.1)**	33.2(25.8-35.4)*#
	γδ TCR+	7.1(4.5-15.8)	14.1(6.6-26.6)***	10.7(7.1-14.1)#
LP-crypt	CD4+	49.9(44.0-78.5)	53.1(38.2-69.9)	61.3(45.6-74.2)
	CD8+	33.6(16.8-43.0)	32.8(20.7-48.2)	29.7(18.0-39.8)
	γδ TCR+	14.2(4.7-22.4)	16.0(6.2-21.2)	9.2(7.8-14.6)
LP total	CD4+	55.4(42.5-63.3)	49.3(38.9-68.3)	59.4(54.7-61.9)
	CD8+	35.5(28.0-49.5)	35.4(19.3-42.1)	31.6(25.3-34.3)
	γδ TCR+	8.6(6.2-16.7)	14.5(6.5-24.6)**	10.4(7.4-12.9)##
EA-villus	CD4+	0.0(0.0-2.6)	1.4(0.0-22.9)	0.5(0.0-1.6)
	CD8+	87.2(77.8-93.6)	82.7(65.9-91.5)*	76.1(51.8-86.5)*
	γδ TCR+	12.1(5.2-22.2)	14.7(7.4-34.2)	23.8(11.9-47.4)*
EA-crypt	CD4+	0.6(0.0-7.0)	1.0(0.0-15.4)	1.4(0.0-9.3)
	CD8+	75.7(63.6-87.4)	78.1(46.2-93.8)	60.4(27.1-86.4)
	γδ TCR+	21.6(12.6-36.4)	17.5(6.3-42.6)	34.2(11.4-72.9)
EA total	CD4+	0.3(0.0-2.3)	1.5(0.0-21.7)	0.8(0.0-3.3)
	CD8+	87.2(76.4-91.9)	82.3(62.4-89.8)	73.0(48.5-82.6)
	γδ TCR+	12.7(8.1-23.3)	15.6(8.2-36.5)	26.9(14.2-50.6)
Villus total	CD4+	36.7(26.0-49.5)	34.8(22.6-60.8)	44.3(31.2-47.6)
	CD8+	54.0(42.9-61.6)	48.1(27.4-61.8)	42.9(33.0-51.5)**
	γδ TCR+	7.7(6.6-16.9)	15.3(8.9-28.9)**	14.0(9.2-23.4)
Crypt total	CD4+	36.2(29.7-49.0)	39.4(30.8-60.0)	54.5(29.3-67.6)
	CD8+	46.9(34.8-52.9)	42.6(25.9-56.3)	31.3(24.3-44.6)*
	γδ TCR+	16.3(10.3-25.7)	16.0(8.6-25.8)	13.1(8.2-26.3)
Total cells	CD4+	37.0(29.2-46.8)	35.0(24.3-60.5)	46.7(30.8-55.3)*
	CD8+	52.0(44.0-58.8)	47.5(26.8-60.4)	40.6(30.8-50.0)**
	γδ TCR+	9.5(8.7-16.5)	15.0(8.8-27.8)*	13.2(9.9-21.9)

Figure 3.5a: Lepromatous case (sheep P6). Photomicrograph of $CD4^+$ immunoperoxidase staining of ileum with lepromatous type lesions. Large macrophages with foamy cytoplasm are present in the lamina propria, and positively stained cells are situated around these. Representative field (x100).

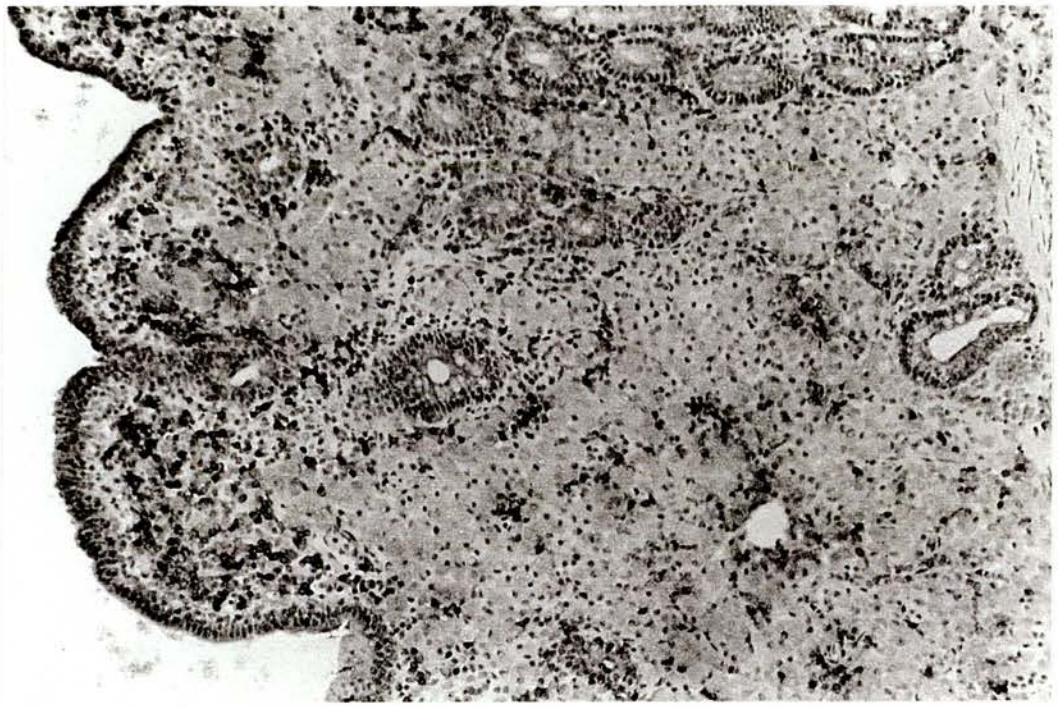


Figure 3.5b: Lepromatous case (sheep P16). Higher power photomicrograph of $CD4^+$ cells in ileal villus. Immunoperoxidase (x200).

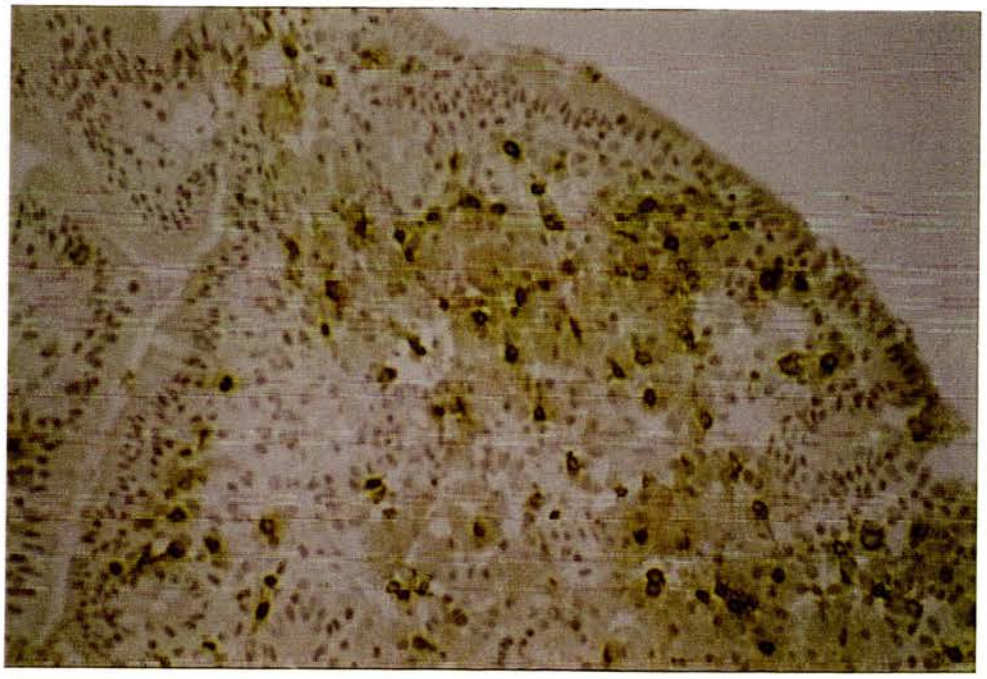


Figure 3.6a: Lepromatous case (sheep P6). Photomicrograph of $CD8^+$ immunoperoxidase staining in ileum with lepromatous type lesions. Positively stained cells are visible mainly in association with the epithelium. Representative field (x100).

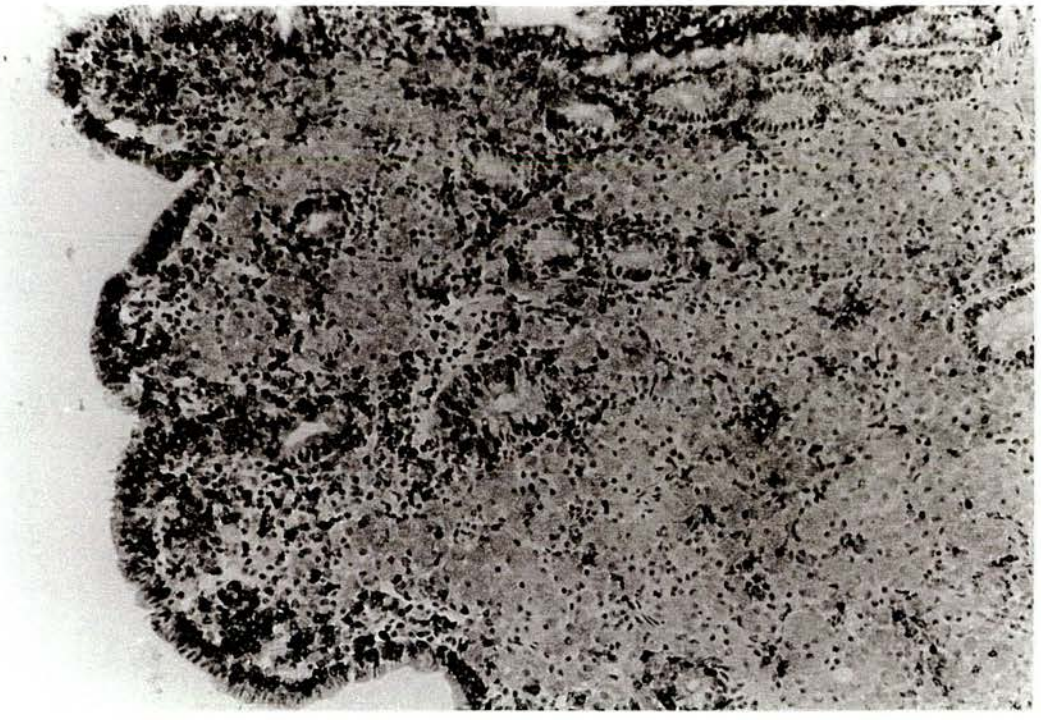


Figure 3.6b: Lepromatous case (sheep P16). Higher power photomicrograph of $CD8^+$ cells in villus. Immunoperoxidase (x200).

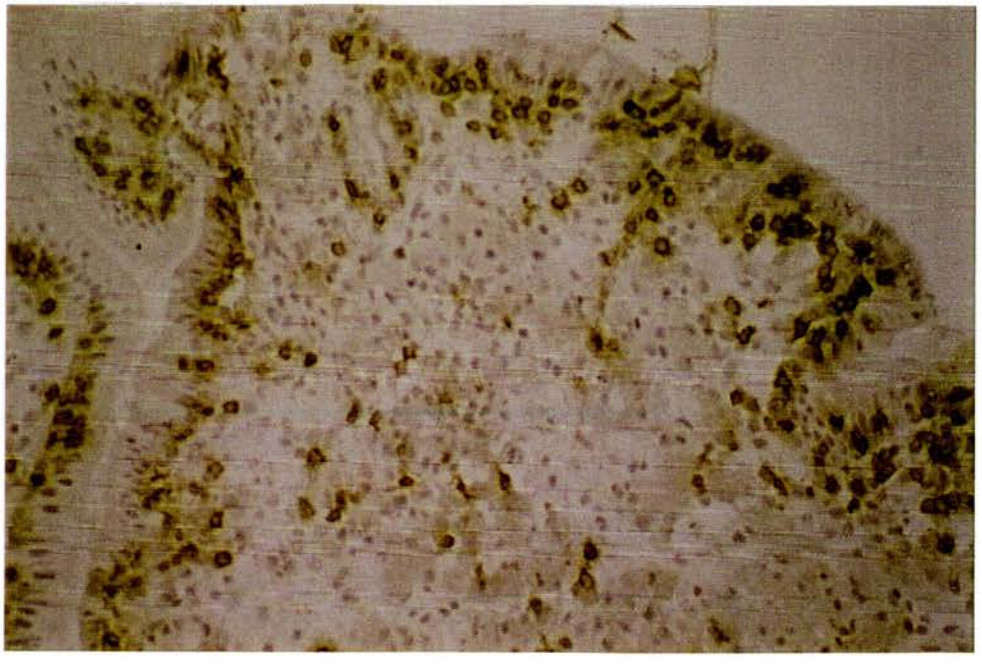


Figure 3.7a: Lepromatous case (sheep P6) Photomicrograph of γδ TCR^+ immunoperoxidase staining in ileum with lepromatous type lesions. Positively stained cells are evident throughout the mucosa. Representative field (x100).

Figure 3.7b: Lepromatous case (sheep P16). Higher power photomicrograph of γδ TCR^+ cells in villus. Immunoperoxidase (x200).

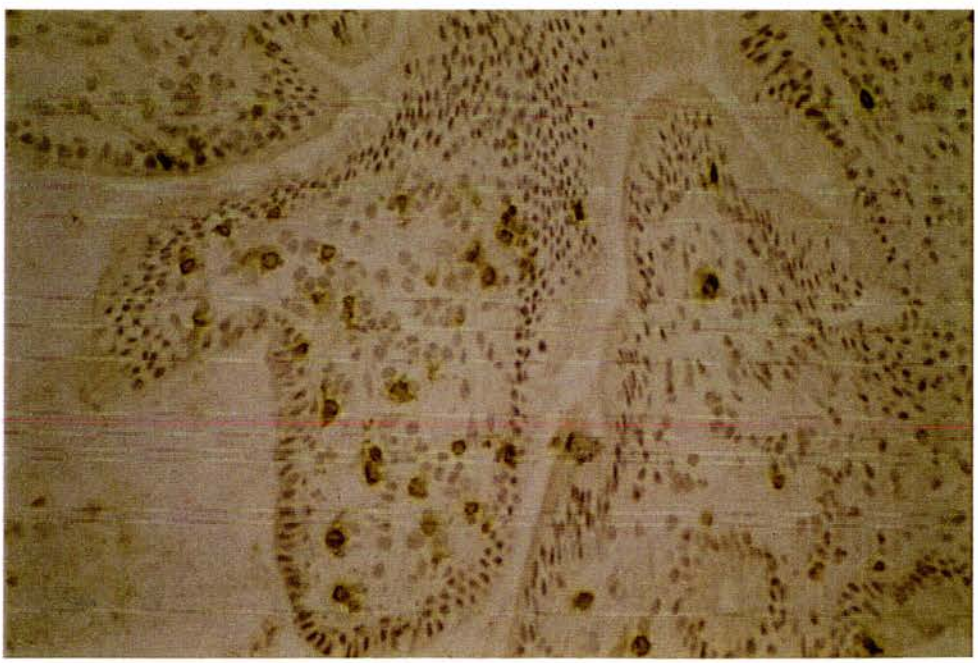


Figure 3.8a: Tuberculoid case (sheep P19). Photomicrograph of $CD4^+$ cells immunoperoxidase staining of ileal mucosa. Increased density of lymphoid is evident, with many positively stained cells visible in the lamina propria. Representative field (x100).

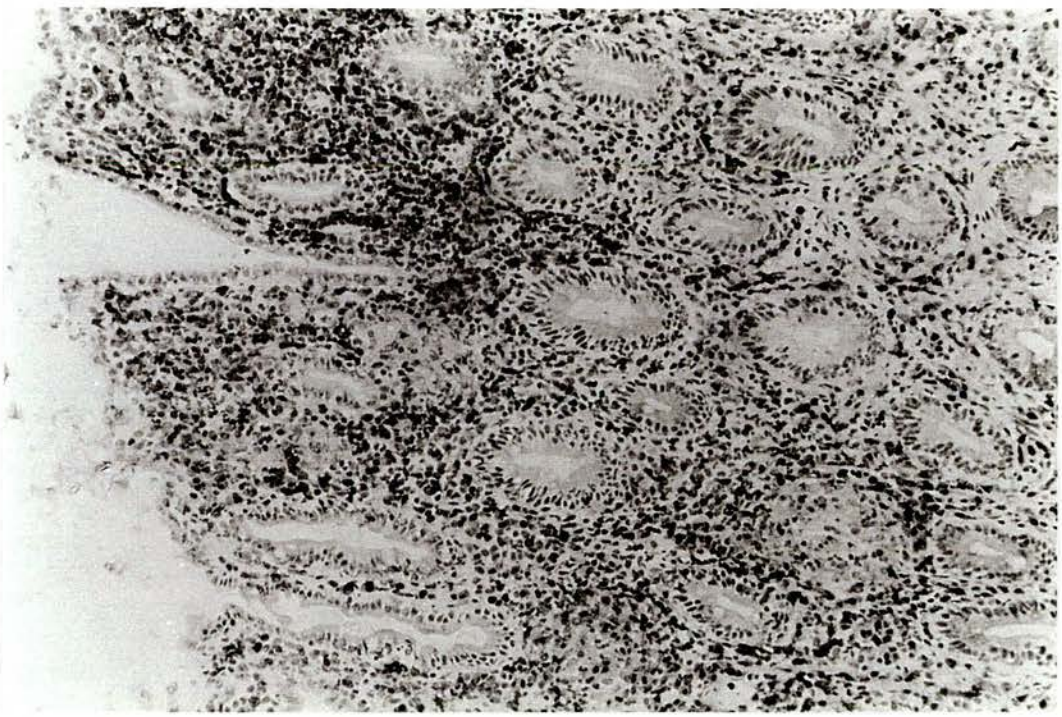


Figure 3.8b: Tuberculoid case (sheep P25). Higher power photomicrograph of $CD4^+$ cells in villus. Immunoperoxidase (x200).

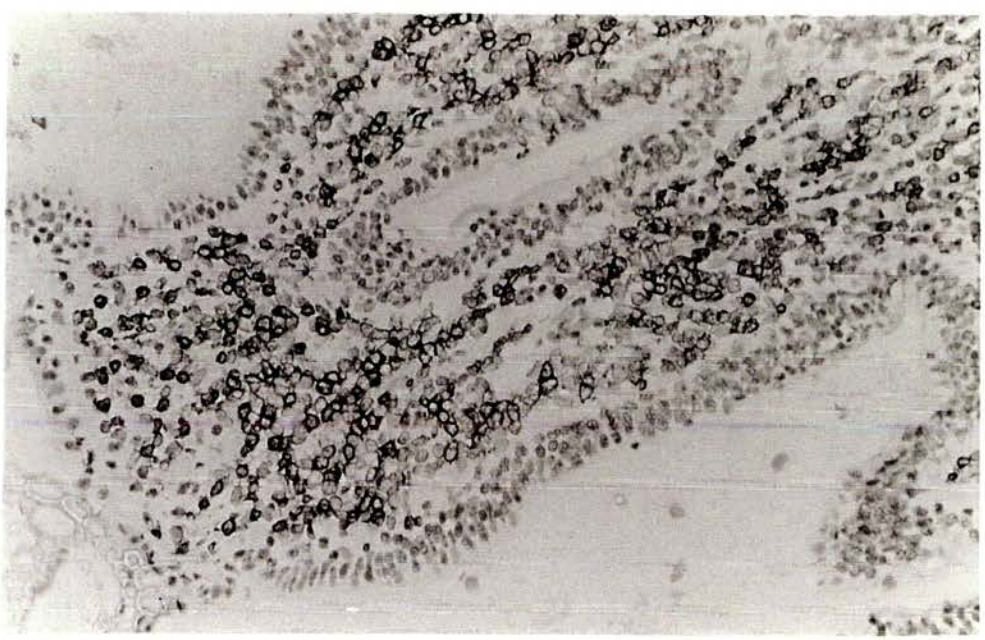


Figure 3.9a: Tuberculoid case (sheep P19). Photomicrograph of $CD8^+$ immunoperoxidase staining in ileal mucosa. High density of lymphoid-cell nuclei is apparent in the lamina propria, although $CD8^+$ cells appear to be fewer in number than $CD4^+$ cells (figure 3.8). Representative field (x100).

Figure 3.9b: Tuberculoid case (sheep P25). Higher power photomicrograph of $CD8^+$ cells in ileal villus. Immunoperoxidase (x200).

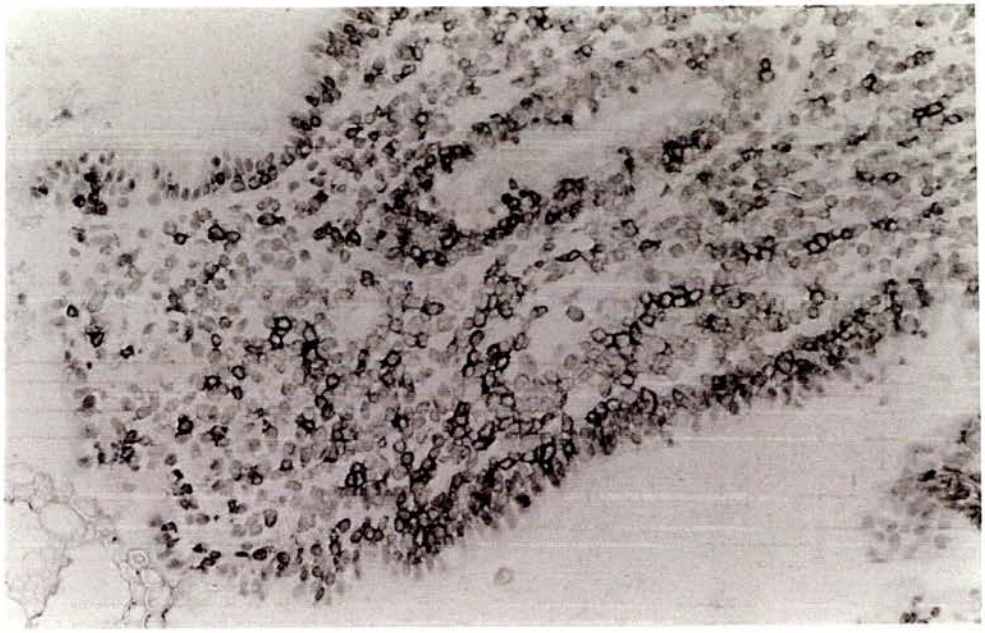


Figure 3.10a: Tuberculoid case (sheep P19). Photomicrograph of γδ TCR^+ immunoperoxidase staining in ileal mucosa. Positively stained cells are visible throughout the mucosa, and in lower numbers than $CD4^+$ and $CD8^+$ cells. Representative field (x100).

Figure 3.10b: Tuberculoid case (sheep P25). Higher power photomicrograph of γδ TCR^+ staining cells in ileal villus, with positively stained cells in the lamina propria and epithelium. Immunoperoxidase (x200).

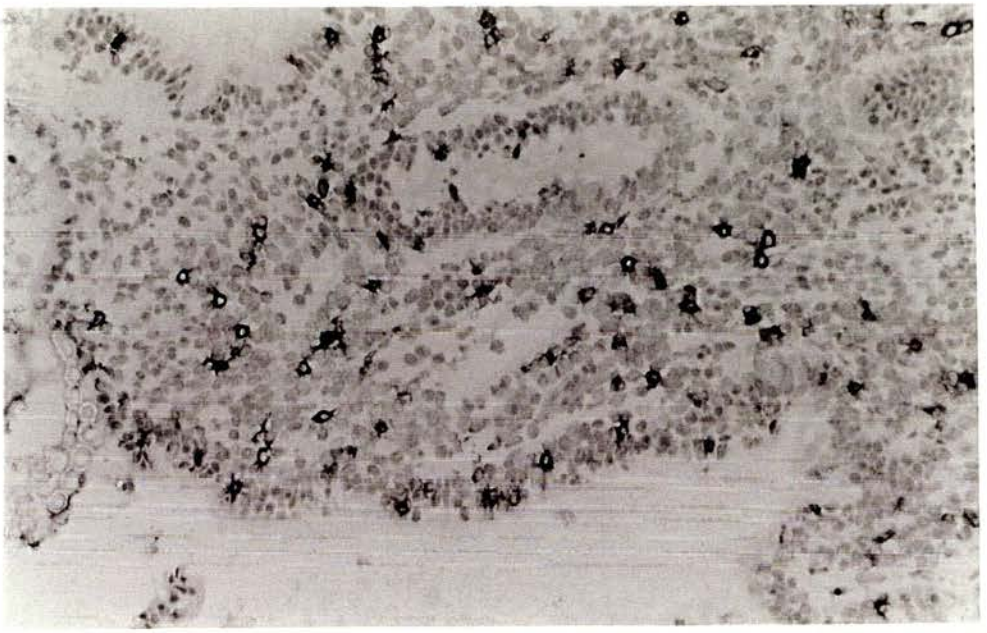
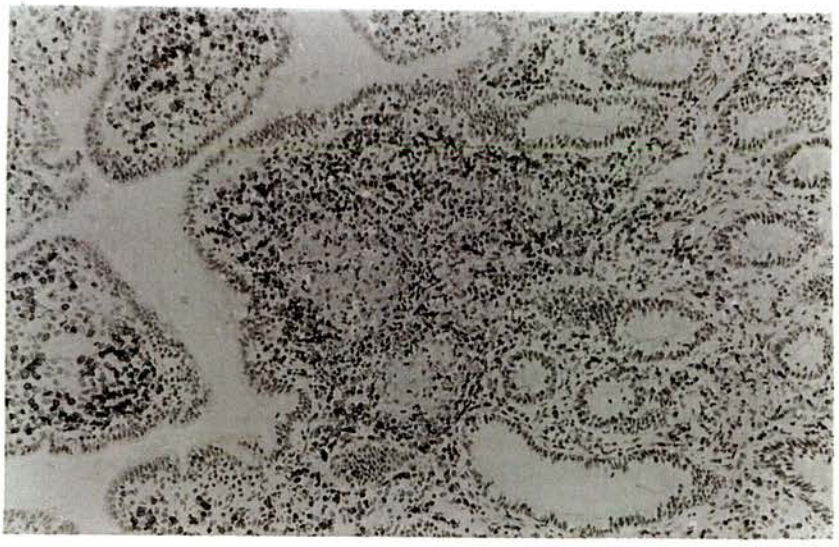


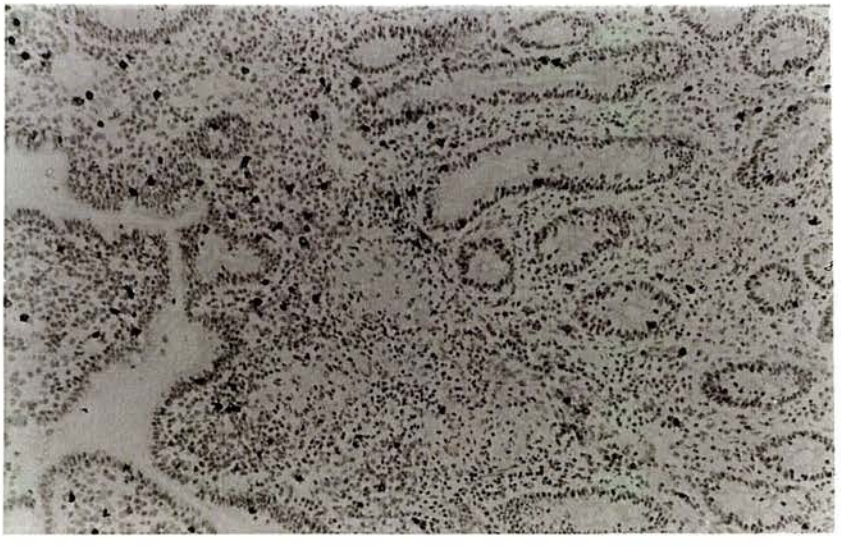
Figure 3.11: Lepromatous case (P14). Three photomicrographs of a granulomatous lesion in ileum. No distinctive pattern of subset distribution in association with focal granulomas is apparent. a: $CD4^+$ cells; b: $CD8^+$ cells; c: $\gamma\delta$ TCR^+ cells. Immunoperoxidase (x100).

a



b

c



($P<0.01$). In addition, lepromatous cases had a higher density of $\gamma\delta$ TCR^+ cells in this compartment compared with both tuberculoid cases ($P<0.05$) and control animals ($P<0.001$).

The only significant differences noted in epithelial-associated cells were an increase in the villus relative percentage of $\gamma\delta$ TCR^+ cells with a corresponding decrease in $CD8^+$ cells in tuberculoid cases ($P<0.05$), and a decrease in the percentage of $CD8^+$ cells in lepromatous compared with control animals ($P<0.05$). This latter decrease in the lepromatous $CD8^+$ percentage may be a function of the margination of $CD4^+$ cells seen as a result of space-occupying aggregations of macrophages. Overall, for total epithelial cells there was no significant difference in EAL between the control, lepromatous and tuberculoid groups.

In the villus compartment, the only significant differences were a lower median percentage of $CD8^+$ cells in tuberculoid and a higher percentage of $\gamma\delta$ TCR^+ in lepromatous compared with control villi (both $P<0.01$). For crypt cells there was no significant difference except for a decrease in the relative percentage of $CD8^+$ cells in the crypts of tuberculoid cases compared with normal animals ($P<0.05$), although the corresponding increase in $CD4^+$ cells was not statistically significant at $P=0.055$. No other significant differences were observed in the crypt lymphocyte population, which appeared to confirm that villi were more affected than crypts in paratuberculosis infection.

3.3.4 Ratios of T-cell subsets

Median cell counts were expressed as ratios, and are included in table 3.7. The CD4:CD8 ratios were calculated for each animal for total cells counted and for the

Table 3.7: Medians of cell counts expressed as $CD4^+$: $CD8^+$: $\gamma\delta$ TCR^+ ratios.

Cells	*Control*	*Lepromatous*	*Tuberculoid*
LPL	5.5 : 4.1 : 1	3.4 : 2.2 : 1	5.8 : 3 : 1
EAL	1 : 87 : 14.3	1 : 30.6 : 6.6	1 : 65.5 : 27.5
Crypt	2.3 : 2.8 : 1	2.4 : 2.7 : 1	3.6 : 2.3 : 1
Villus	3.9 : 5.9 : 1	2.3 : 3.1 : 1	2.8 : 2.9 : 1
Total	3.4 : 4.9 : 1	2.4 : 3.0 : 1	3.1 : 2.7 : 1

Table 3.8: CD4:CD8 ratios. Significant differences from the control group are denoted * ($P<0.05$).

CD4:CD8	*Control*	*Lepromatous*	*Tuberculoid*
	median (min,max)	*median (min,max)*	*median (min,max)*
Villus LP	1.47 (0.76,2.23)	1.51 (0.86,4.97)	1.76 (1.56,2.33)*
Villus total	0.68 (0.46,1.15)	0.70 (0.36,2.22)	1.09 (0.61,1.32)*
Crypt total	0.80 (0.56,1.41)	0.88 (0.56,2.32)	1.78 (0.66,2.78)*
Total	0.72 (0.50,1.06)	0.71 (0.40,2.26)	1.18 (0.62,1.64)*

Table 3.9: CD4+CD8:$\gamma\delta$TCR ratios. Significant differences from the control group are denoted ** ($P<0.01$), and * ($P<0.05$).

CD4+CD8:$\gamma\delta$	*Control*	*Lepromatous*	*Tuberculoid*
	median (min,max)	*median (min,max)*	*median (min,max)*
Villus LP	13.07 (5.34,21.00)	6.11 (2.76,14.09)**	8.39 (6.09,13.07)*
Villus total	11.93 (4.90,14.27)	5.55 (2.47,10.26)**	6.21 (3.28,9.89)*
Crypt total	5.15 (2.89,8.72)	5.26 (2.88,10.60)	6.81 (2.81,11.26)
Total	9.54 (5.07,10.54)	5.73 (2.60,10.35)*	6.61 (3.57,9.06)

various compartments for all three groups of animals (table 3.8). For control and lepromatous animals the median total CD4:CD8 ratios were less than one, due to the predominance of $CD8^+$ cells; however, in tuberculoid animals the median CD4:CD8 ratio was greater than one due to a higher proportion of $CD4^+$ cells. In tuberculoid animals, the median CD4:CD8 ratio was significantly increased compared with non-infected animals ($P<0.05$) for all compartments examined and for total cells. No significant differences were found between lepromatous and control animals nor between lepromatous and tuberculoid animals.

Ratios were calculated for $\gamma\delta$ TCR^+ T cells versus non gamma-delta subsets (table 3.9). Analysis of variance for the total CD4+CD8:$\gamma\delta$ TCR ratios for the three groups of animals showed significant variance between these samples at $p<0.01$ for total cells counted, and $p=0.001$ for total villus cells. Two sample analysis showed significant decreases in the ratios for total cells ($P<0.05$), villus cells and villus lamina propria cells ($P<0.01$) between control and lepromatous groups, and between control and tuberculoid groups for villus cells and lamina propria villus cells only ($P<0.05$). No significant differences were found between the lepromatous and tuberculoid groups.

3.3.5 Intraepithelial lymphocytes

Analysis of the IEL populations of all three groups of animals revealed that this population consists of $CD8^+$ and $\gamma\delta$ TCR^+ cells, with negligible numbers of $CD4^+$ IEL being present (table 3.10). Higher densities of $CD8^+$ IEL were noted in the ileal epithelium of tuberculoid cases compared with animals of both the control and lepromatous groups ($P<0.01$). The median of the total $\gamma\delta$ TCR^+ IEL counts was found to be higher in this group also, but proved not to be statistically significant. The

Table 3.10: Medians of total IEL counts (in 10 lengths of villus epithelium per section). Significant differences from the control group are denoted ** (P<0.01), and * (P<0.05), and difference between the infected groups by ## (P<0.01).

IEL	*Control*	*Lepromatous*	*Tuberculoid*
	median (min,max)	*median (min,max)*	*median (min,max)*
$CD4^+$	0.0 (0, 2)	0.0 (0, 3)	0.0 (0, 1)
$CD8^+$	17.5 (13, 31)	22.5 (16, 33)	56.5 (42, 90)**##
$\gamma\delta TCR^+$	13.5 (6, 36)	11.0 (6, 36)	29.0 (6, 63)
CD8:$\gamma\delta$TCR ratio	1.13 (0.68,4.00)	2.17 (0.58,4.17)*	1.76 (1.02,13.0)

CD8:γδ TCR ratios were calculated for the IEL count data, and that of the lepromatous group was found to be significantly higher than that of the control group ($P<0.05$).

3.4 DISCUSSION

The distribution and localisation of intestinal immune cells in these normal sheep appears to be similar to that described for pigs (Vega-López *et al.*, 1993), and calves (Parsons *et al.*, 1993). Both of these studies utilised immunohistochemistry, and a similar pattern of distribution of $CD4^+$ cells in the central LP area, and $CD8^+$ and γδ TCR^+ T cells in association with the intestinal epithelium was described by these investigators. In calves, however, there appeared to be higher percentages of $CD4^+$ cells in the epithelium. CD4:CD8:γδTCR ratios were also different in calves but this may be due to the age of the animals, as young animals are expected to have a relatively higher percentage of γδ T cells (Hein and Mackay, 1991). The findings of this study concur with the published data that lamina propria lymphocytes are predominantly $CD4^+$, whereas in the intraepithelial compartment $CD8^+$ lymphocytes are the most frequent cells.

The relative percentages in this study did not take account of the possibility that some cells may co-express CD8 and the γδ TCR. However this is unlikely to be highly significant since Gyorffy *et al.* (1992) in an analysis by flow cytometry described only 0.2% $CD8^+$ γδ TCR^+ cells in jejunal Peyer's patch, and 0% in ileal lymph and PBL. The only compartment where $CD8^+$ γδ TCR^+ usage may be significant is in IEL, in which 4.3% of cells were found to co-express these molecules (which represented 25% of γδ TCR^+ IEL). CD4 and CD8 molecules are rarely co-expressed, and likewise nor are CD4 and γδ TCR (Gyorffy *et al.,* 1992).

The phenotypic populations of lymphocytes in the ileal lesions of paratuberculosis appeared to be different from the population in the ileum of normal, non-infected sheep. The presence of two distinct, polar histological forms in the spectrum of paratuberculosis lesions suggests that different immune mechanisms are involved in the pathogenesis of each form. Tuberculoid type lesions in the intestine had increased densities of all T-cell subsets. This increase confirms the histopathological finding that tuberculoid lesions are associated with marked lymphoid infiltrate. The infiltrate was predominantly $CD4^+$ in nature, with a decrease in the relative percentage of $CD8^+$ cells. There is some evidence in support of the functional subdivision of ruminant $CD4^+$ helper cells into Th1 and Th2 subsets (Brown *et al.*, 1993; Brown *et al.*, 1994), although this different cytokine profile may not be as distinct and mutually exclusive in ruminants as in mice, with evidence that ovine lymphocytes produce low levels of IL-10 and IFNγ constitutively (Keating, 1995). The lesions seen may be due to $CD4^+$ cell activity similar to that described as a Th1 type response, resulting in efficient intracellular killing of the organism by infected macrophages and an increase in T-cell proliferation and differentiation associated with the lymphokine IL-2 in particular. This would be consistent with the observed paucibacillary nature of the tuberculoid lesions. However, such a Th1 type response may also result in an associated delayed-type hypersensitivity reaction and consequent immunopathology. $CD4^+$ cells have been shown to be the predominant infiltrating T-cell subset in tuberculoid lesions of human leprosy (Van Voorhis *et al.*, 1982; Modlin *et al.*, 1988) and this type of lesion is associated with high levels of IFN γ production (Salgame *et al.*, 1991). In tuberculoid leprosy lesions mRNA for the Th1 cytokines IFNγ and IL-2 has been shown to predominate, whereas mRNA for the Th2 cytokines IL-4, IL-5 and IL-10 was predominant in lepromatous lesions (Yamamura *et al.*, 1991).

In lepromatous type lesions it was noted that, compared with non-infected control gut, there was a decrease in the $CD4^+$ and $CD8^+$ cell densities, which may be due to the presence of high numbers of large, infected macrophages acting as a space occupying lesion. This would appear to confirm that lepromatous cases are associated with an infiltrate which predominantly consists of macrophages. The absolute numbers of the $\gamma\delta$ TCR^+ subset did not change significantly and there was no significant change in the relative percentages of $CD4^+$ and $CD8^+$ subsets compared with normal sheep. However, there was a significant increase in the relative percentage of the $\gamma\delta$ TCR^+ subset. In lepromatous lesions of leprosy the infiltrating cell type is predominantly $CD8^+$ (Van Voorhis *et al.*, 1982; Modlin *et al.*, 1988), however in lepromatous lesions of paratuberculosis there was no significant change in the $CD8^+$ subset compared with control animals.

Lepromatous type pathology appears to correlate directly with higher levels of antibody production in ovine paratuberculosis (chapter 2) suggesting that in lepromatous cases, the persistence of the pathogen may be explained by a misdirected, mainly Th2 type of response, resulting in inappropriate antibody production. It has been shown that $CD8^+$ cells from lepromatous leprosy lesions can be activated to suppress $CD4^+$ T-cell proliferation in vitro (Modlin *et al.*, 1986). On the basis of phenotype only, no significant difference in the CD4:CD8 ratios between lepromatous and non-infected animals was found, however this was not an assessment of the functional activity of the lymphocyte subsets.

Gamma-delta TCR^+ T cells are a prominent subset within the ruminant immune system (Hein and Mackay, 1991), and have been shown to have a greater repertoire of antigen receptors in sheep than in mice (Walker *et al.*, 1994) which may suggest that sheep may have a higher dependence on this particular subset than other species in respect to certain antigens. Gamma delta TCR^+ T cells are known to be involved in the

protective response to mycobacterial infections (Janis *et al.*, 1989; Modlin *et al.*, 1989; Augustin *et al.*, 1989; Inoue *et al.*,1991; Havlir *et al*, 1991) and to respond to a range of mycobacterial antigens (Rust and Koning, 1993) and stress proteins (Haregewoin *et al.*, 1990; Kaufmann and Kabelitz, 1991). The γδ TCR^+ T-cell subset has also been implicated in granuloma formation in mycobacterial disease (Mombaerts *et al.*, 1993), and $CD8^+$ cells have been reported to form a mantle around granulomas in leprosy lesions (Cooper *et al.*, 1989), however preliminary observations on the lymphocyte subsets associated with focal granulomas did not reveal any apparent patterns of accumulation of cells of any phenotype at these sites. Human γδ T-cell cultures have been shown to produce the Th1 type cytokines IFNγ and IL-2 *in-vitro* in response to activation by *M. tuberculosis* (Follows *et al.*, 1992) and murine γδ T cells in response to *Listeria monocytogenes* (Ferrick *et al.*, 1995). Their presence in higher relative percentages in the lepromatous cases may be a result of the increased numbers of mycobacteria in these multibacillary lesions, as the γδ TCR^+ subset has been shown to be expanded by live *M. tuberculosis* (Boom *et al.*, 1992), in contrast with heat killed bacteria, which $CD4^+$ cells were able to recognise but γδ TCR^+ cells could not. Modlin *et al.* (1989), described increased numbers of γδ TCR^+ cells in the lesions of reversal reactions, and at the site of lepromin injection, but not in tuberculoid or lepromatous lesions, and so considered these cells to be associated with inflammatory lesions.

A cytotoxic immunoregulatory function on $CD4^+$ T cells has been described for γδ TCR^+ peripheral blood lymphocytes in bovine paratuberculosis (Chiodini and Davis, 1992). Likewise, the γδ TCR^+ cell cytotoxicity was found also to be subject to downregulation by $CD8^+$ cells (Choidini and Davis, 1993). Increased numbers of γδ TCR^+ T cells in the intestine may be an indication that they play an active role in the local immune response to paratuberculosis.

The two histological forms of ovine paratuberculosis described were associated with different lamina propria lymphocyte populations. This suggests that distinct immune mechanisms are involved in the pathogenesis of each form. The effector functions of these populations require characterisation, and further investigations on the proliferative responses and cytokine production of cells are described in subsequent chapters.

CHAPTER FOUR

PHENOTYPIC CHARACTERISATION OF THE LYMPHOCYTE SUBSETS IN PARATUBERCULOSIS BY FLOW CYTOMETRIC ANALYSIS

4.1 INTRODUCTION

The distribution and densities of T-cell subsets in ileum were described in chapter 3, as determined by immunohistochemical analysis. Some of the MAbs initially assayed for the immunohistochemical study were not effective in staining tissue sections, and in the case of B cell and MHCII staining the resultant positive staining was not readily quantifiable by cell counting. For these cell surface markers in particular, flow cytometry was chosen to be the preferred method for quantitative study. Flow cytometry allowed study of the proportions of lymphocyte subsets, and not an assessment of the absolute numbers or densities of these subsets, which has been indicated for gut T cells by immunohistochemical staining. The aims of this study were to isolate lymphoid cells from sheep intestine in order to facilitate studies on the immune responses of intestinal lymphocytes in paratuberculosis, and to further examine the phenotype of lymphocytes in the mucosal compartment of normal sheep, and determine any differences in this population in *Map* infection. Ileum was chosen as it was seen to be the section of intestine most consistently affected in paratuberculosis. To this end, a gut digestion protocol was devised which would permit isolation of viable lymphocytes for study. A suitable method is described which gave satisfactory yields. Cell isolation methods were modified and the isolated populations examined by flow cytometry, and cells were then made available for culture and further studies. Mesenteric lymph node lymphocytes (MLNL) were also isolated and studied, since afferent lymph drains to these nodes from the gut mucosa,

and MLN were found to be the most frequent secondary site of paratuberculosis lesions (chapter 2). PBL were also isolated from the blood of infected and control animals. Cells isolated from these tissues were analysed phenotypically by flow cytometry using a panel of monoclonal antibodies specific for sheep T-cell markers, sIg (B cells), MHCI and MHCII subgroups. In combination with MAb technology, flow cytometry has proved to be an important tool for the characterisation of cell populations, and a means of determining the density of expression of cell surface markers in addition, in particular MHCII antigens which are upregulated on T-cell activation (Hopkins *et al.,* 1993).

Phenotypes of LPL have been investigated by a number of authors in various species. Mega *et al.*, (1991), investigated the murine LP subsets by flow cytometry and described predominance of $CD4^+$ lymphocytes (24.5%) and lower percentages of $CD8^+$ cells (15.3%) and 5% double negative (DN) $CD3^+$ cells. Janossy *et al.*, (1980), Selby *et al.*, (1983) and James *et al.*, (1986) reported similar proportions in human LP. Zeitz *et al.*, (1991), reviewed LPL phenotypes of humans and non-human primates. In this review, the authors collated flow cytometric data from their previous publications for human LPL, and described a population comprising 53% (20-90) $CD4^+$, 35% (14-38) $CD8^+$, and 3% (1-5) $\gamma\delta$ TCR^+ cells. 93% (62-99) of LPL were found to be CD45RO positive, compared with 41% of PBL, and only 10% (2-16) $CD45RA^+$ (PBL 33%). CD25 was expressed on the surface of 18% (7-33) of LPL compared with only 3% of PBL. HML-1 ($\alpha E\beta 7$ integrin) was found on 38% (9-54) of LPL compared with only 2% of PBL, and on the majority of IEL. These findings describe a compartmentalised population of cells which is distinct from circulating lymphocytes on the basis that it is composed of a high percentage of memory cells, and also high levels of intestine specific antigens such as $\alpha E\beta 7$- integrin (HML-1) (Cerf-Bensussan *et al.*, 1987) which is considered to be a marker of lymphocyte activation in the gut (Zeitz *et al.,* 1991). A higher degree of activation is evident in

LPL compared with PBL, signified by increased CD25 and HML-1 expression, and MHCII upregulation (Zeitz *et al.*, 1988). High percentages of memory cells are found in the lamina propria compartment (Zeitz *et al.*, 1991). Intestinal memory cells in sheep have been shown to be highly gut tropic in their recirculation, and unlike naive cells are L-selectin negative (Mackay *et al.*, 1992). The $CD4^+$ and $CD8^+$ proportions appeared to be reversed in porcine LP (Rothkötter et al., 1994).

Hopkins *et al.* (1993) and Dutia *et al.* (1993) showed that MHCII was expressed on a proportion of sheep T cells, and the expression of different class II homologues (products of different MHC gene loci) was progressive and dependent on age and immunological compartment. A progression of MHCII subgroup expression was described on secondary antigen activation, with the phenotype of naive cells (and in foetal animals) being DQ^-DR^-, progressing through DQ^-DR^+ to DQ^+DR^+ in activated/memory T cells. A twofold increase in DR^+ and fourfold increase in DQ^+ cells was seen in efferent lymph after secondary antigen challenge. B cells, however, did not show altered levels of expression of MHCII in secondary responses to antigen. On this basis, cell-surface expression of MHCII homologues were examined in IEL, LPL, MLNL and PBL.

4.2 MATERIALS AND METHODS

4.2.1 Animals

A total of 17 control sheep, (of which 7 were Scottish Blackface, 3 Dorset cross, 2 Finnish Landrace cross, 1 Cheviot, 1 Texel, 1 Suffolk cross, and 1 not recorded), 10 tuberculoid cases (4 Scottish Blackface, 3 Cheviot, 2 Greyface, and 1 not recorded), and 15 lepromatous cases (6 Scottish Blackface, 6 Cheviot, 1 Suffolk

cross, and 2 not recorded) were included in this study. All animals were female and adult, with the exception of 2 control lambs (C44 and C45) which were 11 and 14 months respectively. Animals showing significant intercurrent disease were excluded from the study, and FACS (fluorescence-activated cell sorting) data which appeared to be invalid due to technical problems such as FACScan malfunction, or loss of cells were also excluded from the data set used for statistical analysis. For individual observations, the number of animals included is given in the tables of results.

4.2.2 Collection of tissues

All tissues for cell isolation were collected fresh at necropsy (chapter 2), with the exception of peripheral whole blood which was collected ante-mortem by percutaneous jugular venipuncture into either a 50 ml tube containing 500 IU of heparin sodium anticoagulant solution (Leo Laboratories, Princes Risborough, UK), or into vacutainer tubes containing 10 IU ml^{-1} of heparin sodium.

4.2.3 Media

The following media were used in the isolation of lymphocytes from ileum and mesenteric lymph nodes: HBSS (Gibco BRL Life Technologies, Paisley). HBSS without Calcium and Magnesium (Gibco BRL) supplemented with 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin (HBSS-CMF). RPMI-1640 (Gibco BRL) supplemented with 10 % Foetal Calf Serum (FCS) (Ato Tek, Hereford), 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin, and 2mM L-glutamine (RPMI-FCS).

4.2.4 Monoclonal antibodies

Monoclonal antibodies used were from stocks held within the Department of Veterinary Pathology, and are shown in table 4.1.

4.2.5 Preparation of PBL from whole blood

Lymphocyte isolation from whole blood was performed as soon as possible after collection, generally within one hour. Whole blood was diluted by addition of an equal volume of 0.9% NaCl solution and mixed thoroughly by inversion. A 16 ml aliquot of the diluted blood solution was carefully layered onto 8 ml of 'Lymphoprep' (Nycomed, Oslo, Norway) in a 25 ml sterile plastic universal container. Tubes were prepared in duplicate or triplicate as necessary. The tubes were then spun at 800 x g for 20 minutes at room temperature in a centrifuge (Beckman, Palo Alto, CA), and allowed to stop without brake assistance. Resultant bands of cells were removed from the sample/medium interface using a Pasteur pipette. Cells were resuspended in HBSS and washed three times by centrifugation to ensure that any residual Lymphoprep was removed, and finally resuspended. The concentration of cells in suspension was measured in an haemocytometer, viability assessed by exclusion of Trypan Blue stain (2%) (Sigma), and the final concentration adjusted as necessary.

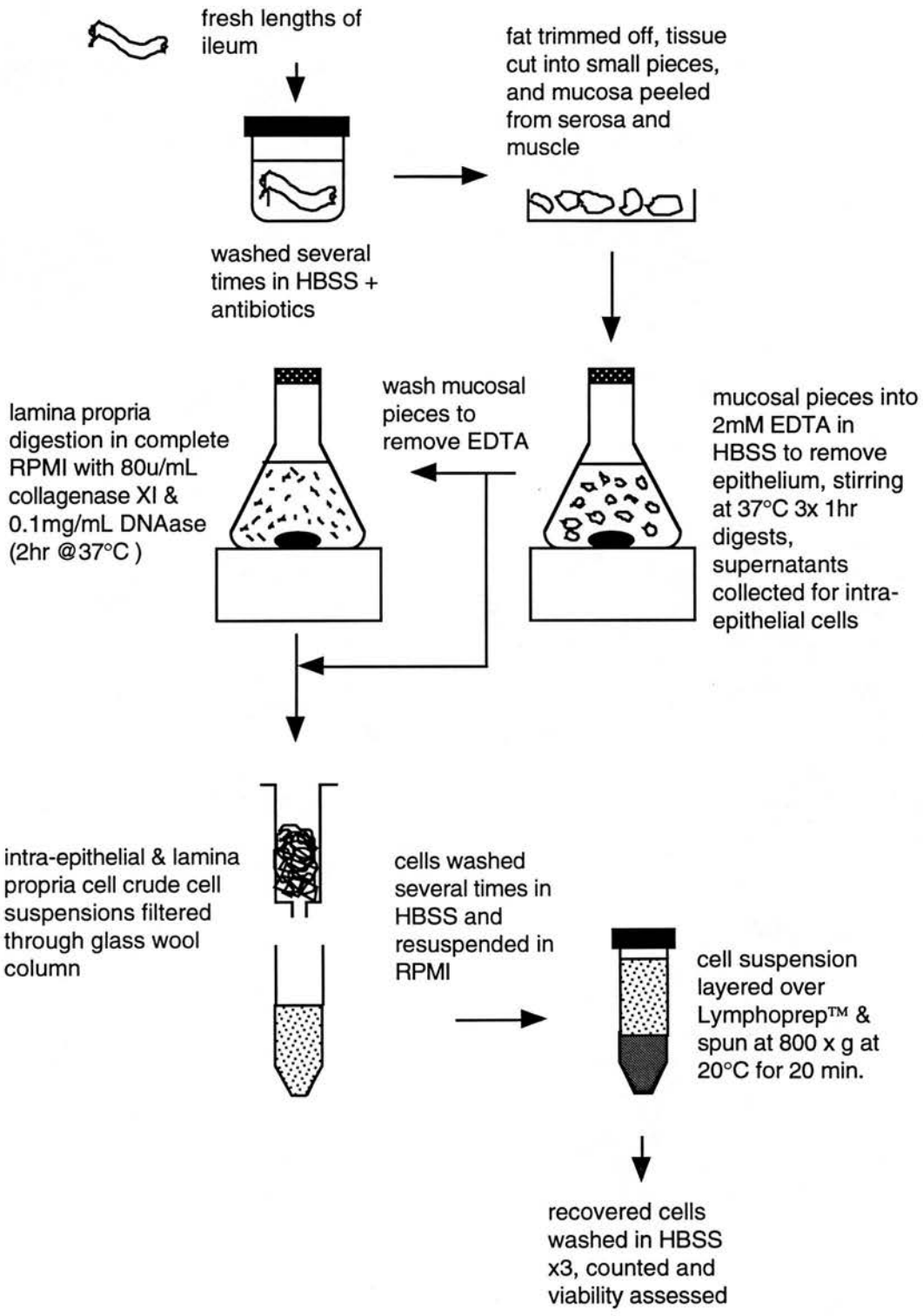
4.2.6 Gut cell isolation

Intestinal lamina propria cells and intraepithelial cells were isolated using a procedure modified in particular from those described for bovine LP and IEL by Nagi and Babiuk (1987), and Clough and Dean (1988), and for the isolation of LP and IEL

cells from rat and murine guts by Lyscom and Brueton (1982), and Davies and Parrott (1981), respectively. These workers described isolation of IEL and LPL populations based on enzymatic digestion of lamina propria with prior removal of the epithelium by chelation or mechanical disruption. A variety of adaptations of these isolation procedures were attempted, ranging from mechanical disruption of the mucosa by 'mincing' the tissue through mesh screens, to enzymatic digestion using a variety of enzymes (such as Dithiothreitol (DTT) (Sigma) for the removal of mucus, and Dispase (Sigma) and Collagenase Type V (Sigma)). Isolation procedures were microscopically checked by preparing cytospins of digest supernatants and HE staining of routine sections of tissue fragments. Resultant cell suspensions were then purified using a number of density gradient centrifugation protocols. Percoll gradients (Pharmacia, Uppsala, Sweden), and Metrizamide (Nycomed) were tried before Lymphoprep was chosen on the bases of the most satisfactory numbers and type of cells yielded and time invested. The isolation procedure which was finally adopted is shown schematically in figure 4.1, and described herein. All data in this chapter were derived using the following final protocol only.

Lengths of ileum measuring approximately 8-10 cm were collected fresh at necropsy, opened longitudinally, and placed into sterile, ice-cold HBSS in a covered receptacle. Care was taken to handle tissues in such a manner to prepare cell suspensions that were non-contaminated despite starting with tissue which is potentially contaminated by gut contents. Potential infectivity of materials was also considered and as a consequence all cell isolation work was carried out in a Class II Microbiological Safety Cabinet (Envair, Rossendale, Lancs). All glassware and tissue culture materials used were sterile, and had been previously silicon-coated using Sigmacote (Sigma) in order to reduce adhesion by macrophages which were required for study by a simultaneous project. Aseptic technique was used in the handling of all

Figure 4.1: Schematic diagram showing lamina propria cell isolation procedure.



media, tissues and cell suspensions. In a sterile environment, the tissue was washed several times in HBSS-CMF progressively through three sequential beakers.

The lengths of ileum were prepared by trimming off fat, and cutting the ileum into pieces of approximately 4 cm^2. The mucosa was then stripped from the underlying muscular and serosal layers by scraping with a scalpel blade. Mucosal pieces were collected into a 250 ml conical flask containing 100 ml HBSS-CMF and 2mM EDTA (ethylenediaminetetraacetic acid) (pH readjusted to pH 7.2) which had been pre-warmed to 37°C. The flask was covered and the contents stirred using a magnetic stirrer for three hours in an incubator at 37°C to remove the intestinal epithelium, the media being changed at 1 hourly intervals. Supernatants were collected from the first EDTA digest, decanted through a sterile nylon-mesh tea strainer and used for the preparation of IEL. Cells were pelleted by centrifugation at 450 x g for 5 minutes and then washed thoroughly to remove all traces of EDTA by resuspending in ice-cold HBSS and centrifugation. The wash process was repeated twice, and cells reserved on ice until purification. After three hours, the HBSS-EDTA medium was decanted off and the mucosal fragments washed by stirring in RPMI-FCS (pre-warmed to 37°C) for 20 minutes. RPMI-FCS was then decanted and discarded and the fragments of ileum were enzyme-digested by stirring in 100 ml pre-warmed RPMI-FCS containing 80 U ml^{-1} Collagenase Type XI (Sigma) and 0.1mg ml^{-1} Deoxyribonuclease II (DNase) Type V (Sigma) for 1 hour at 37°C. LP cell suspension supernatants were decanted after 1 hour through a sterile nylon-mesh tea strainer, collected, and cells pelleted by centrifugation at 650 x g for 5 minutes.

4.2.7 Purification of isolated cells

In order to remove particulate material, dead cells, and also importantly, mucus, each LPL and IEL cell suspension was filtered down a glass wool column which had been silicon coated using Sigmacote as before, and pre-wetted with HBSS. Filtered cells were then washed three times in HBSS. Cell concentration and viability were assessed (as above) at this stage before Lymphoprep purification. Lymphoprep purification was carried out as described above using the same relative volumes of cell suspension and medium. A maximum of 10^8 cells were loaded onto each Lymphoprep tube as higher numbers than this resulted in a decreased yield of purified cells. Purified cells were harvested and washed three times in HBSS by centrifugation, and resuspended in RPMI-FCS. Cells were again counted in an haemocytometer and viability reassessed. Tubes containing cells were kept on ice at all times until required.

4.2.8 Isolation of MLNL from mesenteric lymph node

RPMI-FCS medium containing 50 U ml^{-1} collagenase XI (Sigma) and 0.1 mg ml^{-1} DNase II Type V (Sigma) was prepared fresh and pre-warmed to 37°C (RPMI-enzyme medium). MLN were resected at necropsy and immediately collected into ice-cold sterile HBSS and the receptacle covered. In a sterile environment, MLN were trimmed of fat and peri-adnexal structures, and cut into small pieces. Pieces of MLN were then gently teased apart in a sterile microbiological dish containing a few ml of RPMI-enzyme and dissected into smaller fragments using two sterile hypodermic needles. The resultant suspension of dissected cells and MLN fragments were emptied into a 125 ml conical flask containing 75 ml of RPMI-enzyme medium and incubated at 37°C stirring for 45 minutes. Cell suspension was then decanted, purified by filtration down a silicon coated glass wool column, and washed three times in ice-cold

HBSS as described above. Cell concentration and viability were assessed as before. Cells were then purified by Lymphoprep centrifugation and prepared for use as previously described.

4.2.9 Preparation of cells for FACS analysis

Cells for FACS analysis were aliquoted as required to allow 1 x 10^6 cells per analysis tube, centrifuged and supernatant discarded and resuspended in PBA wash buffer, (PBS with 1% Bovine Serum Albumin (BSA), heparin and sodium azide 0.1% (appendix 4.1)) at a concentration of 2 x 10^7 cells per ml. A 50 µl aliquot of cell suspension (equivalent to 1 x 10^6 cells) was added to a FACS round-bottomed mini test tube (Sterilin, Hounslow), and 25 µl of the monoclonal antibody at working dilution was added (table 4.1). A single tube containing a sample of isolated cells was left unstained for use as a negative control, and to provide a sample for setting a 'live gate' for the lymphocyte population. Such an unstained control tube was prepared for each population of cells analysed from each animal. One further tube of cells for each group was stained using a 1:100 dilution of normal mouse serum instead of MAb, and this tube used in the analysis as a negatively stained control for estimation of non-specific binding. Cells were incubated with MAb for 30 minutes at 4°C, after which cells were washed by adding 600 µl of wash buffer (PBA) to each tube and the tubes were then centrifuged at 450 x g for 5 minutes. The supernatant liquid was discarded, the cells resuspended in 600 µl of PBA and the wash process repeated. 25 µl of a 1:100 predetermined optimal dilution in PBA of FITC-conjugated $F(ab')_2$ fragment rabbit anti-mouse immunoglobulins (Dako, Glostrup, Denmark), or rabbit anti-rat immunoglobulins (Dako) depending on the species specificity of the primary MAb, were added to the tube and the cells resuspended. This cell suspension was incubated for 15 minutes at 4°C in the dark, before undergoing two further washes as before.

Table 4.1: Monoclonal antibodies recognising ovine (Ov) leukocyte antigens.

Antibody	*Specificity*	*Isotype*	*Dilution*	*Reference*
SBUT4	OvCD4	IgG2a	1:1000	Maddox *et al*, 1985
SBUT8	OvCD8	IgG2a	SS	Maddox *et al*, 1985
86D	Ovγδ TCR	IgG1	SS	Mackay *et al*, 1989
CC15	Bovine T19	IgG2a	SS	Howard *et al*, 1989
ST1	OvCD5	IgG2a	SS	Beya *et al*, 1986
35A-1	OvCD2	IgG1	SS	Giegerich *et al.*, 1989
VPM8	OvB cell sIg	IgG1	SS	Jones, 1988
VPM18	OvCD45	IgG1	SS	Hopkins & Dutia, 1990
VPM19	OvMHCI	IgG1	SS	Hopkins & Dutia, 1990
VPM36	OvMHCII DQα	IgG1	SS	Dutia *et al*, 1990
VPM54	OvMHCII DRα	IgG1	SS	Dutia *et al*, 1994
SW73.2	OvMHCII panβ chain	IgG2b (rat)	SS	Hopkins *et al*, 1986

SS - hybridoma culture saturated supernatant

Table 4.2 Lamina propria cell yields from whole gut and mucosa.

LP Cell Yields	*Cells/g. gut ($x10^6$)*		*Cells/g. mucosa ($x10^6$)*	
	n=	*median (range)*	*n=*	*median (range)*
Non-infected	5	4.6 (2.5-10.5)	8	10.2 (2.9-22.3)
Infected (all)	13	8.2 (3.0-15.3)	6	12.6 (5.1-28.8)
Lepromatous	8	7.0 (3.0-15.3)	3	8.2 (5.1-28.8)
Tuberculoid	5	8.4 (3.8-13.0)	3	16.9 (6.4-18.9)
Goats	3	3.7 (0.8-4.6)	3	11.0 (1.9-13.3)
Deer*	0		6	8.5 (6.3-13.9)

* not discussed

The cells were finally resuspended in 500 µl of PBA and kept at 4°C in the dark until analysed using the FACScan flow cytometer. When analysis was not possible on the same day as preparation, cells were fixed by resuspending in 200 µl PBA and adding 200 µl 1% paraformaldehyde solution (appendix 4.1).

4.2.10 FACS analysis

FACS analysis was performed using a Becton Dickinson FACScan flow cytometer (Becton Dickinson, Oxford) with a 488 nm argon laser in operation. Data were acquired and saved using 'Consort 30' software (Becton Dickinson). Initially, a control unstained sample of cells was run through the machine in order to visualise the forward scatter (FSC) and side scatter (SSC) patterns of the total cell population. From this FSC by SSC dot-plot profile, the lymphocyte population, characterised by low FSC and SSC, was selected and a 'live gate' manually set surrounding this population. This gate also excluded dead cells on the basis of FSC and SSC. The details of the gate setting were recorded, and applied to all analyses of positively stained cells. Once the gate was set, samples were run through the machine with the green fluorescence detector (FL-1) then in operation to detect the population of cells that exhibited positive staining by the antibody-FITC complexes (FITC emission 520nm). Gates were reset between each animal and each tissue examined, and were periodically checked during the data acquisition. 10^4 gated cells were counted and analysed. Data were analysed using 'Consort 30' and 'Lysis' software programmes. Positively stained cells were enumerated by overlaying the histogram for cells stained only by normal mouse serum. Results were expressed as percentage of positively stained cells, and in addition for MHCII, the mean channel number of fluorescence intensity was chosen as an indicator of the density of cell-surface antigen. Mean channel was chosen in preference to mode (peak) channel since the histogram curves

were generally not consistent with a normal distribution curve, and so, the mean channel was considered to be a more appropriate parameter.

4.2.11 Statistics

Normal distribution of data was not able to be confirmed for all samples and so non-parametric tests were adopted for all the statistical analyses. The Mann-Whitney rank sum test was applied to analyses between two groups, and Kruskall-Wallis tests were used to determine one-factor analysis of variance between three groups. Statistical analysis was performed using 'Minitab for Windows' software (Minitab Inc., State College, PA.) and significance was considered to be $P<0.05$.

4.3 RESULTS

4.3.1 Gut digestion

The method described above provides adequate yields of intraepithelial and lamina propria cells of viability suitable for cellular studies. Table 4.2 indicates the approximate yields of lamina propria cells achieved, which were calculated from figures noted for approximate total gut tissue weights and for approximate weight of stripped mucosal tissue prior to processing. Viability was highly acceptable, with the median percentage of viable cells in total LP cell counts being 74% (n=19, range 60-93%) before Lymphoprep purification. After purification, which was always carried out prior to use of cells in further analysis or studies, median viability rose to 93%, (n=19, range 87-99%). Isolation of cells from MLN likewise gave good yields with acceptable viability. The median MLN total cell yield was 5.5×10^8 cells (n=14, range

2.2-14 x 10^8) prior to Lymphoprep purification and 6 x 10^7 (n=22, range 2.2-23 x 10^7) after purification. Again viability was good with the median percentage of viable cells 81% before (n=10, range 72-92%) rising to 93% (n=15, range 85-98%) after purification.

4.3.2 Phenotypes of intraepithelial cells

Median percentages and ranges of positively stained IEL are included in table 4.3, and figure 4.2 shows representative FACScan dot-plot and histograms obtained for IEL. The predominant lymphocyte subset in the IEL population was $CD8^+$ in all three groups of animals. Significantly higher percentages of $CD4^+$ cells were present in the IEL fraction isolated from the group of animals with tuberculoid type lesions than control ($P<0.01$) and lepromatous ($P<0.05$) groups, and this group also had significantly lower percentages of $\gamma\delta$ TCR^+ and $T19^+$ cells (86D and CC15 positively stained cells) than control ($P<0.01$) and lepromatous ($P<0.05$) groups.

4.3.3 Phenotypes of LPL

Median percentages and ranges of positively stained LPL are included in table 4.4, and figure 4.3 shows dot-plot and histograms representative for LPL. $CD4^+$ cells were the highest percentage subset of LPL in all three groups of animals, and although not statistically significant, the percentage of $CD4^+$ cells appeared to be higher in tuberculoid cases than in lepromatous ($P=0.09$) and control ($P=0.45$) groups. The tuberculoid group was associated with a significantly lower percentage of $CD8^+$ cells compared with the lepromatous group ($P<0.05$), but the lower median percentage than the control animals was not significant ($P=0.07$). The lepromatous group had a

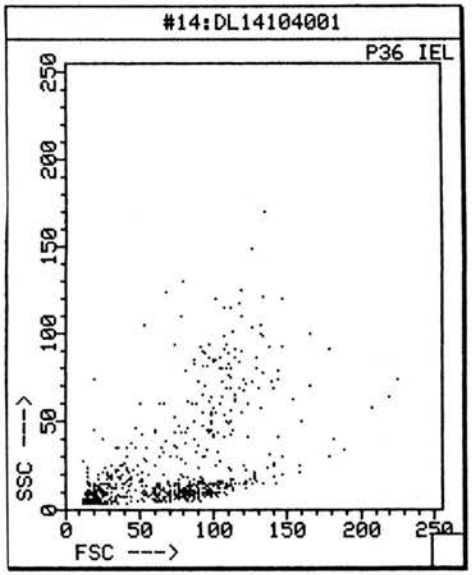
Table 4.3: IEL Percentages of positively stained cells on FACS analysis.

	Control			*Lepromatous*			*Tuberculoid*		
Mab	*N*	*Median*	*Range*	*N*	*Median*	*Range*	*N*	*Median*	*Range*
SBUT4	16	10.34	3.49-26.45	11	12.82	5.99-21.52	6	25.30**#	11.21-57.26
SBUT8	16	60.71	16.46-81.14	11	41.39	0.47-78.68	6	31.35	9.03-66.03
86D	15	8.91	4.54-14.82	11	6.03	3.52-12.65	6	4.55**#	1.75-5.58
CC15	14	5.67	0.46-17.78	10	5.39	2.33-11.74	6	2.64*#	0.57-5.05
ST1	9	9.67	4.12-14.16	9	12.28	5.68-24.16	4	22.63*	11.33-28.93
VPM8	11	8.98	0.52-65.36	7	19.68	2.93-52.95	3	10.85	2.39-23.67
VPM19	14	94.51	78.97-99.53	10	87.93	72.45-97.71	5	94.15	88.18-98.78
SW73.2	3	92.15	79.79-99.35	9	84.76	52.70-95.48	3	87.70	85.35-96.18
VPM36	6	36.06	20.17-75.22	6	49.92	28.12-75.51	4	43.76	26.07-54.66
VPM54	8	67.46	49.21-89.12	7	68.28	29.02-73.70	4	40.97	37.34-70.76

Significant differences from the control group are denoted ** ($P<0.01$) and * ($P<0.05$).

Significant differences of tuberculoid from lepromatous group are denoted and # ($P<0.05$).

Figure 4.2 (opposite): Representative FACScan dot-plot of cells isolated by EDTA digestion, and histograms of ovine (sheep P36) IEL. The panel of MAbs used is represented, and MAb name is indicated on each histogram. The dot-plot is plotted with SSC on the ordinate and FSC on the abscissa. The histograms represent green-fluorescence (FL-1) intensity detected for cells within the lymphocyte live gate. The NMS histogram is included and was overlaid onto MAb-stained samples to allow determination of percentages of positively stained cells.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P36 IEL
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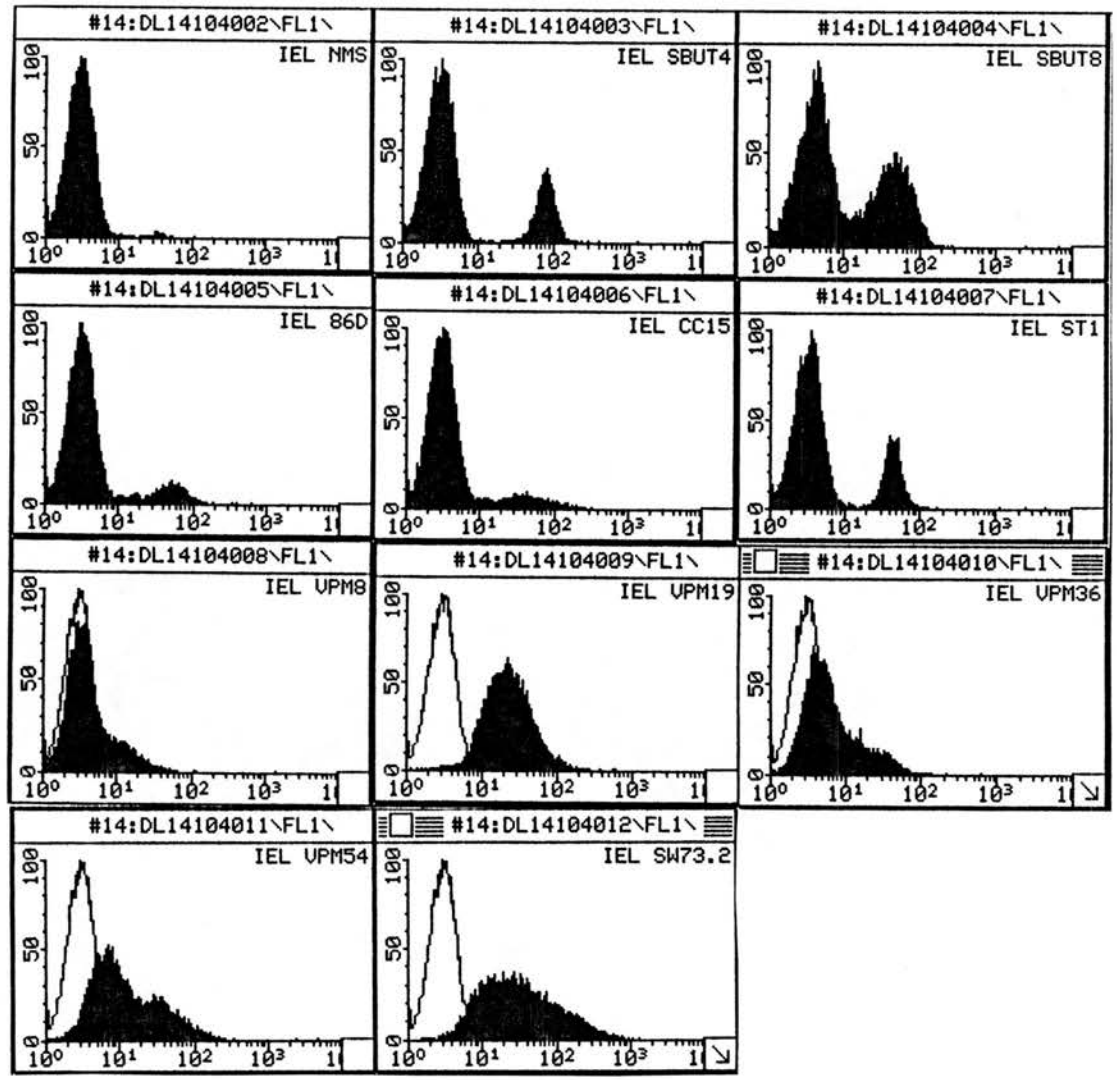
FSC --->


#14:DL14104002\FL1\
IEL NMS
#14:DL14104003\FL1\
IEL SBUT4
#14:DL14104004\FL1\
IEL SBUT8
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IEL 86D
#14:DL14104006\FL1\
IEL CC15
#14:DL14104007\FL1\
IEL ST1
#14:DL14104008\FL1\
IEL VPM8
#14:DL14104009\FL1\
IEL VPM19
#14:DL14104010\FL1\
IEL VPM36
#14:DL14104011\FL1\
IEL VPM54
#14:DL14104012\FL1\
IEL SW73.2

significantly higher percentage of $T19^+$ cells than both control ($P<0.05$) and tuberculoid ($P<0.01$) groups, and although this was reflected in the percentages of $\gamma\delta$ TCR^+ cells, the apparent difference was not significant for the 86D MAb. No significant differences were noted in the percentages of sIg^+ staining cells between the three groups. Median percentages of IEL and LPL which stained positively for CD4, CD8 and $\gamma\delta$ TCR were added, and are represented graphically in figure 4.4.

4.3.4 Phenotypes of MLNL

Median percentages and ranges of positively stained MLNL are included in table 4.5. Figure 4.5 is included to show a dot-plot and histograms representative of those obtained for MLNL. B cells (sIg positive cells) were the most prevalent lymphocyte subset isolated from MLN. The highest percentages of T cells from all three groups were $CD4^+$ lymphocytes, with approximate CD4:CD8 ratios of 2:1. No significant differences between the MLNL population from the three groups of animals were noted, with the exception of the percentages of $\gamma\delta$ TCR^+ and $T19^+$ cells, which were significantly higher in the lepromatous group compared with control animals ($P<0.05$), and although not statistically significant ($P=0.16$ and $P=0.28$ respectively), percentages also appeared to be higher than in tuberculoid cases.

4.3.5 Phenotypes of PBL

Median percentages and ranges of positively stained PBL are shown in table 4.6, and representative FACScan profiles are included in figure 4.6. Both groups of infected animals had significantly lower percentages of $CD8^+$ and $\gamma\delta$ TCR^+ cells and higher percentages of sIg^+ cells than the control group ($P<0.05$). No significant

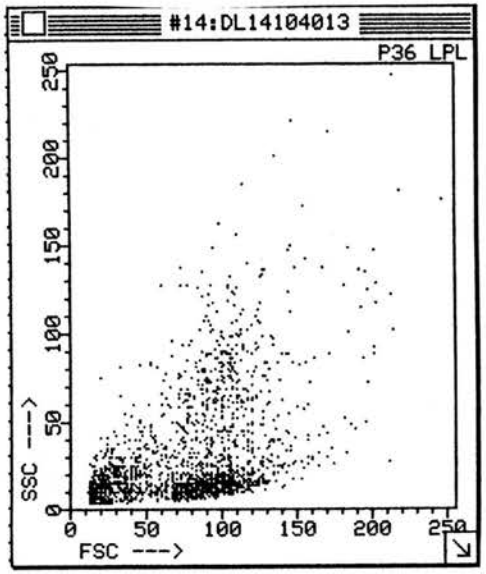
Table 4.4: LPL Percentages of positively stained cells on FACS analysis.

	Control			*Lepromatous*			*Tuberculoid*		
Mab	*N*	*Median*	*Range*	*N*	*Median*	*Range*	*N*	*Median*	*Range*
SBUT4	15	50.99	9.51-64.17	11	36.48	25.00-51.85	7	60.07	20.76-66.15
SBUT8	15	25.33	4.91-46.85	11	28.31	6.90-49.48	7	17.46#	11.92-30.94
86D	15	5.45	1.42-10.41	11	7.09	3.86-15.80	7	4.75	2.67-11.03
CC15	14	3.85	0.53-10.85	10	6.83*	4.87-15.58	7	3.98##	0.98-11.21
ST1	12	48.79	11.60-61.00	9	36.38	7.67-51.55	6	57.83#	32.75-63.32
VPM8	15	5.29	0.52-24.52	11	4.41	1.27-11.25	7	2.96	1.59-8.89
VPM19	15	94.48	60.84-99.35	11	90.93*	44.56-97.01	7	92.17	54.09-96.15
SW73.2	3	68.60	56.80-98.50	9	80.73	60.06-90.14	3	87.14	83.79-88.98
VPM36	2	39.90	21.9-57.9	9	54.35	20.32-84.34	4	46.60	27.9-79.0
VPM54	4	55.90	32.4-79.8	9	51.81	34.05-75.40	6	61.48	22.53-75.01

Significant differences from the control group are denoted * ($P<0.05$).

Significant differences of tuberculoid from lepromatous group are denoted ## ($P<0.01$), and # ($P<0.05$).

Figure 4.3 (opposite): Representative FACScan dot-plot of cells isolated by enzyme digestion, and histograms of ovine (sheep P36) LPL. The panel of MAbs used is represented, and MAb name is indicated on each histogram. The dot-plot is plotted with SSC on the ordinate and FSC on the abscissa. The histograms represent green-fluorescence (FL-1) intensity detected for cells within the lymphocyte live gate. The NMS histogram is included and was overlaid onto MAb-stained samples to allow determination of percentages of positively stained cells. Similar profiles are evident for the ST1 and SBUT4 MAbs which suggests that the aliquot of ST1 used was in fact not OvCD5-specific, but an OvCD4-specific MAb.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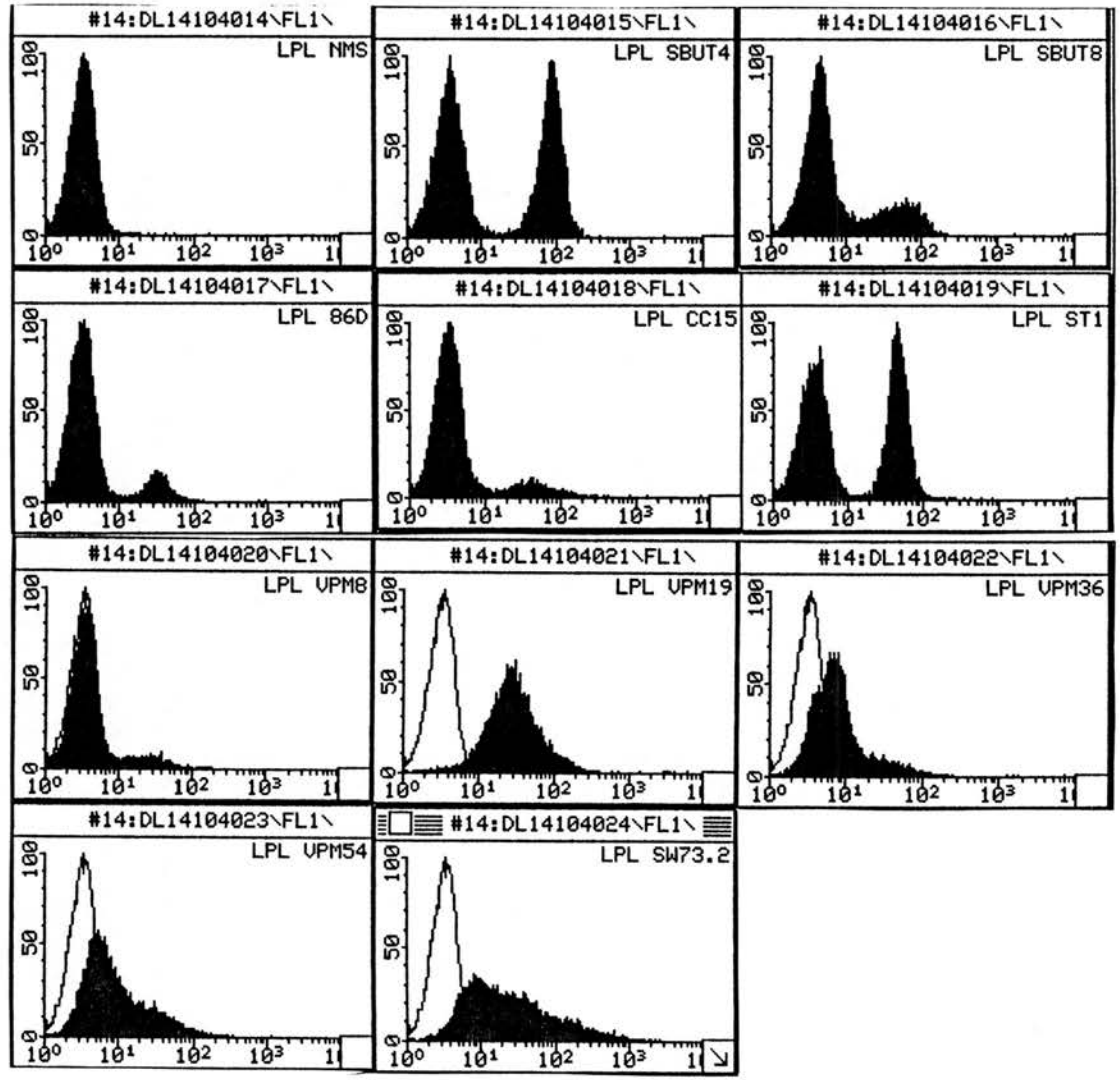
P36 LPL
SSC --->
FSC --->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LPL SBUT4
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LPL SBUT8
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LPL CC15
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LPL ST1
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LPL VPM8
#14:DL14104021\FL1\
LPL VPM19
#14:DL14104022\FL1\
LPL VPM36
#14:DL14104023\FL1\
LPL VPM54
#14:DL14104024\FL1\
LPL SW73.2

Figure 4.4: Graph showing cumulated percentages of positively-stained lymphocytes on FACS analysis of cells isolated from EDTA (IEL) and enzyme (LPL) digests for each group of animals.

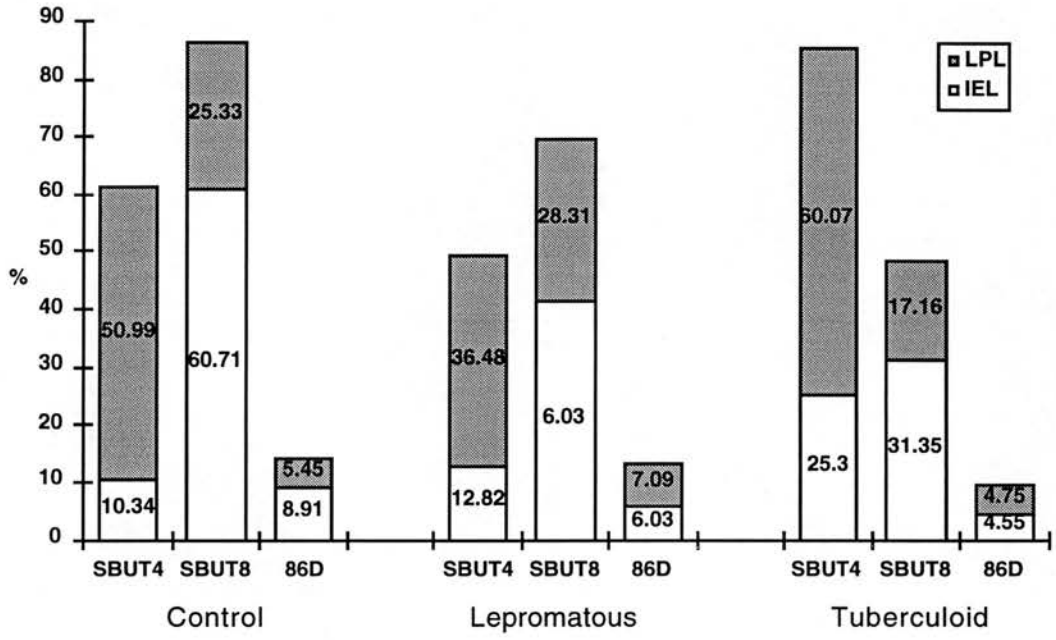
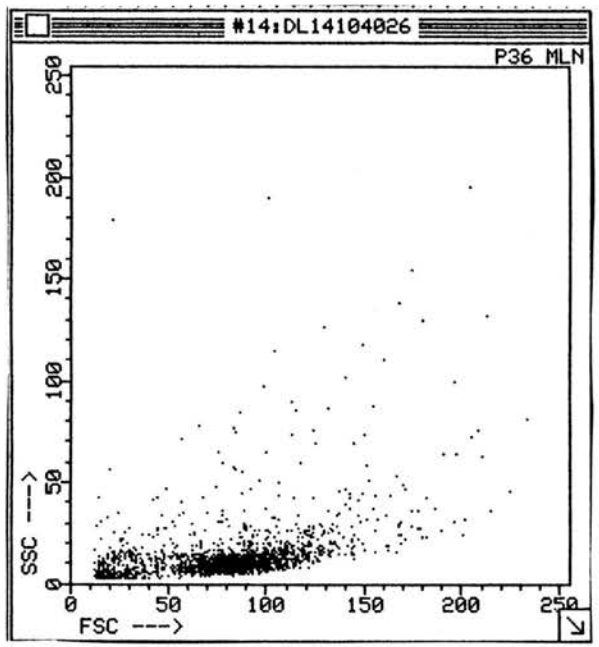


Table 4.5: MLN Percentages of positively stained cells on FACS analysis.

	Control			*Lepromatous*			*Tuberculoid*		
Mab	*N*	*Median*	*Range*	*N*	*Median*	*Range*	*N*	*Median*	*Range*
SBUT4	13	25.33	14.58-40.79	10	28.23	18.26-33.62	6	22.16	20.58-32.96
SBUT8	13	14.74	8.43-26.84	10	13.15	6.85-29.52	6	13.99	8.07-39.92
86D	13	2.43	0.95-6.61	9	4.70*	0.94-9.83	6	2.89	2.02-5.37
CC15	13	2.48	0.51-9.33	10	4.08*	1.64-10.16	6	2.90	0.76-7.84
ST1	10	27.56	17.39-45.85	9	27.73	5.37-31.06	6	20.75	16.52-26.32
VPM8	13	45.62	24.09-64.53	9	39.82	23.28-62.09	5	40.58	20.64-62.41
VPM19	13	96.25	86.89-99.33	9	96.29	84.87-99.21	5	94.06	88.90-96.65
SW73.2	3	92.04	87.01-93.96	9	86.46	71.64-90.76	3	87.01	86.20-89.10
VPM36	13	64.90	44.29-87.54	9	65.56	47.17-75.57	5	49.60	44.32-74.82
VPM54	13	75.00	42.98-91.84	9	70.39	47.17-77.81	5	67.56	39.73-76.90

Significant differences from the control group are denoted * ($P<0.05$).

Figure 4.5 (opposite): Representative FACScan dot-plot of cells isolated by mechanical- and enzyme-digestion of MLN, and histograms of ovine (sheep P36) MLNL. The panel of MAbs used is represented, and MAb name is indicated on each histogram. The dot-plot is plotted with SSC on the ordinate and FSC on the abscissa. The histograms represent green-fluorescence (FL-1) intensity detected for cells within the lymphocyte live gate. The NMS histogram is included and was overlaid onto MAb-stained samples to allow determination of percentages of positively stained cells.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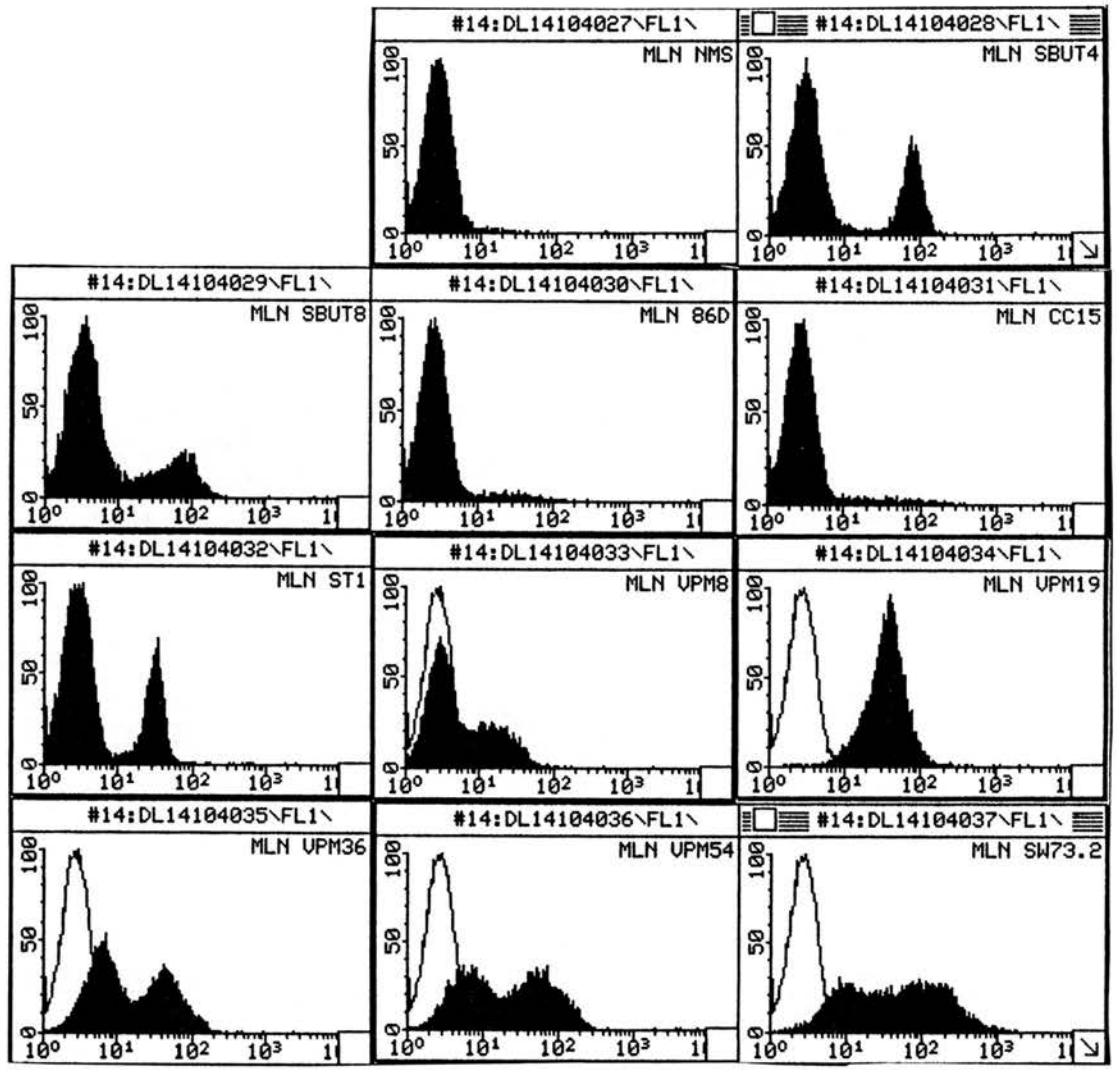
FSC --->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MLN SBUT4
#14:DL14104029\FL1\
MLN SBUT8
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MLN 86D
#14:DL14104031\FL1\
MLN CC15
#14:DL14104032\FL1\
MLN ST1
#14:DL14104033\FL1\
MLN VPM8
#14:DL14104034\FL1\
MLN VPM19
#14:DL14104035\FL1\
MLN VPM36
#14:DL14104036\FL1\
MLN VPM54
#14:DL14104037\FL1\
MLN SW73.2

differences were found between the three groups in the percentages of $CD4^+$ cells. Lower percentages of $CD8^+$ and $\gamma\delta$ TCR^+ cells were noted in animals of the lepromatous group ($P<0.001$), and similarly in animals of the tuberculoid group ($P<0.01$). However, these decreases were most likely due to the corresponding increase in the percentages of sIg^+ cells.

4.3.6 Ratios

CD4:CD8 and CD4+CD8:$\gamma\delta$ ratios are shown in tables 4.7 and 4.8 respectively. Despite the apparent differences, the ratios calculated for LPL of control and infected cases were not significantly different. However, IEL (cells from EDTA digest) from tuberculoid cases had a significantly higher CD4:CD8 ratio than control animals ($P<0.05$), and the CD4+CD8:$\gamma\delta$ ratio was significantly higher than lepromatous and control groups ($P<0.01$). MLNL isolated from the nodes of lepromatous cases displayed significantly lower CD4+CD8:$\gamma\delta$ ratios than control MLNL ($P<0.05$), a consequence of the higher percentages of $\gamma\delta$ TCR^+ cells in the MLNL of these animals. There was no significant difference in the CD4:CD8 ratios. The CD4:CD8 ratios calculated for PBL from control animals were found to be significantly lower than those for both groups of paratuberculosis cases ($P<0.05$), with no difference between the groups of infected animals. Nor was there any difference in the CD4+CD8:$\gamma\delta$ ratios for PBL.

4.3.7 MHC Class II staining

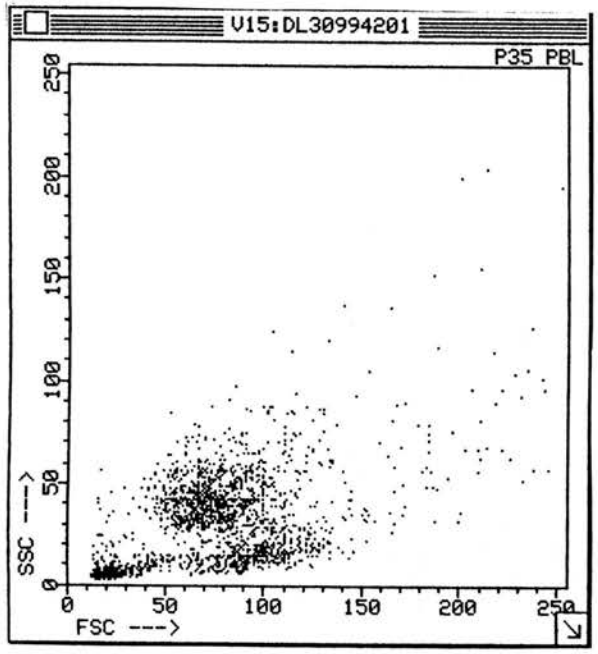
Three MAbs were used for the detection of MHC Class II homologues (table 4.1), and the percentages of cells exhibiting positive staining with these MAbs are

Table 4.6: PBL Percentages of positively stained cells on FACS analysis.

	Control			*Lepromatous*			*Tuberculoid*		
Mab	*N*	*Median*	*Range*	*N*	*Median*	*Range*	*N*	*Median*	*Range*
SBUT4	17	11.75	5.88-23.07	14	9.69	3.20-25.60	9	15.51	2.28-21.98
SBUT8	17	17.48	6.04-43.14	14	8.26***	2.83-19.42	9	8.35**	4.51-16.42
86D	17	7.54	4.54-15.52	13	3.10***	1.26-11.49	9	2.92**	1.43-9.08
CC15	17	6.92	0.82-15.22	11	4.79	2.94-11.16	9	3.40	1.29-13.60
ST1	12	11.10	5.31-18.37	9	4.37*	3.45-18.17	6	14.77	3.28-23.17
VPM8	15	33.07	13.02-72.54	14	59.47*	14.51-85.76	7	54.92*	32.71-88.78
VPM19	17	97.42	82.45-99.66	12	97.73	94.04-99.33	9	97.67	79.15-99.26
SW73.2	6	80.35	62.00-89.90	12	86.19	62.33-93.34	7	87.90	27.46-93.76
VPM36	16	52.37	28.72-72.46	11	72.29**	34.85-89.38	8	74.17*	31.80-89.29
VPM54	16	59.65	46.03-88.07	10	79.44*	49.04-91.12	7	74.65	42.27-91.25

Significant differences from the control group are denoted *** ($P<0.001$), ** ($P<0.01$), and * ($P<0.05$).

Figure 4.6 (opposite): Representative FACScan dot-plot of cells isolated from peripheral blood by Lymphoprep centrifugation, and histograms of ovine (sheep P35) PBL. The panel of MAbs used is represented, and MAb name is indicated on each histogram. The dot-plot is plotted with SSC on the ordinate and FSC on the abscissa. The histograms represent green-fluorescence (FL-1) intensity detected for cells within the lymphocyte live gate. The NMS histogram is included and was overlaid onto MAb-stained samples to allow determination of percentages of positively stained cells.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FSC --->
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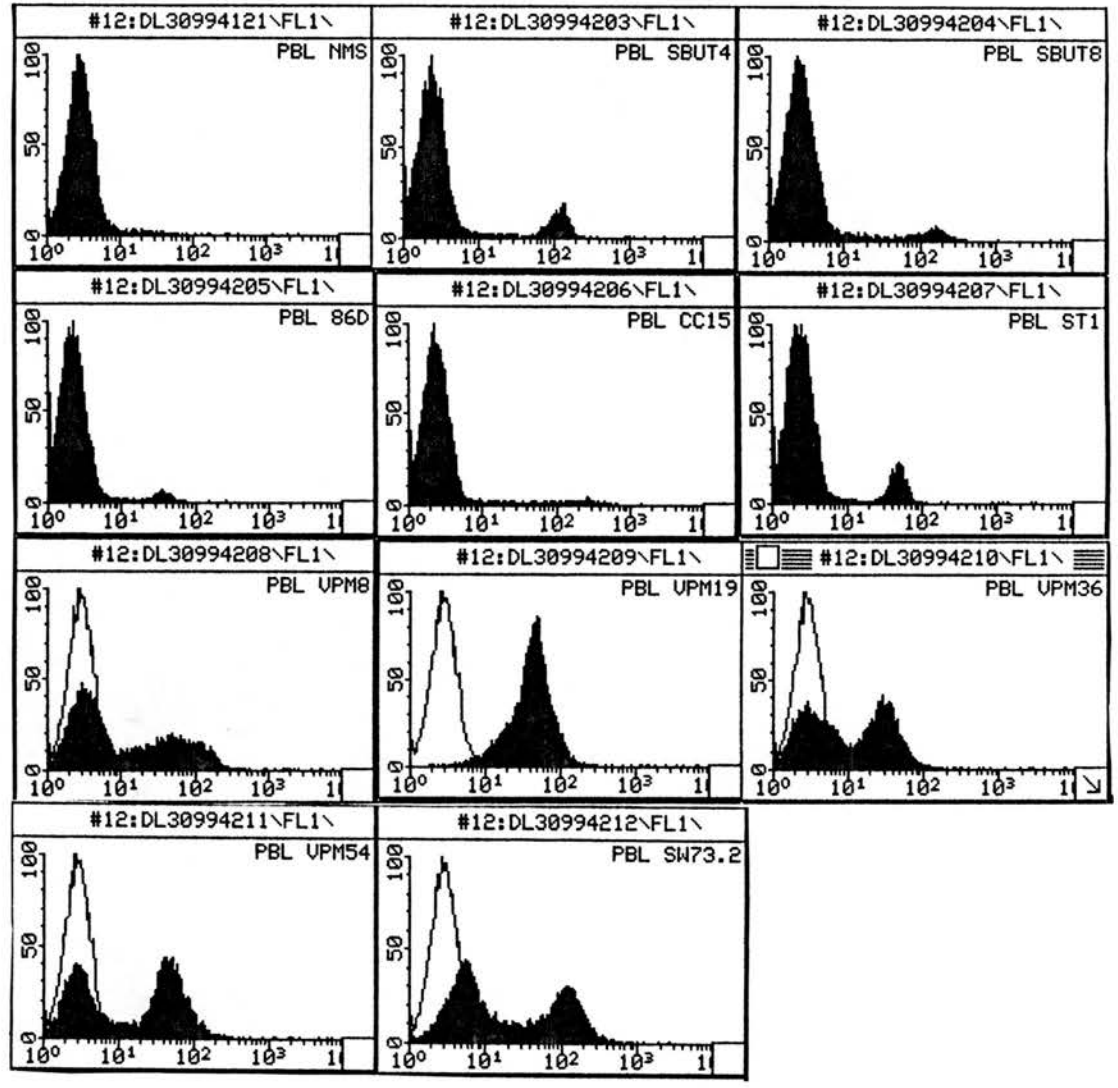
50
100
150
200
250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PBL SBUT4
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PBL SBUT8
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PBL 86D
#12:DL30994206\FL1\
PBL CC15
#12:DL30994207\FL1\
PBL ST1
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PBL VPM8
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PBL VPM19
#12:DL30994210\FL1\
PBL VPM36
#12:DL30994211\FL1\
PBL VPM54
#12:DL30994212\FL1\
PBL SW73.2

Table 4.7: CD4:CD8 ratios calculated from FACS data.

CD4:CD8	*Control*		*Lepromatous*		*Tuberculoid*	
	n=	*median (min,max)*	*n=*	*median (min,max)*	*n=*	*median (min,max)*
LPL	15	2.02 (0.29,4.02)	11	1.28 (0.51,5.08)	7	3.16 (1.11,4.14)
IEL	16	0.17 (0.05,1.54)	11	0.32 (0.10,20.04)	6	1.11 (0.17,6.34)*
MLN	13	1.78 (0.94,3.71)	10	1.83 (0.66,3.20)	6	1.86 (0.68,2.76)
PBL	17	0.59 (0.23,1.95)	14	1.32 (0.46,2.09)*	9	1.37 (0.35,2.39)*

Table 4.8: CD4+CD8:γδ ratios calculated from FACS data.

CD4+8:γδ	*Control*		*Lepromatous*		*Tuberculoid*	
	n=	*median (min,max)*	*n=*	*median (min,max)*	*n=*	*median (min,max)*
LPL	15	13.66 (4.22,23.90)	11	10.16 (5.48,16.04)	7	14.99 (5.29,30.76)
IEL	15	7.88 (2.35,12.33)	11	6.86 (1.73,14.45)	6	14.25 (12.16,19.07)**##
MLN	13	16.96 (6.97,39.33)	9	9.16 (4.76,29.52)*	6	12.96 (5.74,17.17)
PBL	17	4.86 (1.14,8.67)	13	4.52 (2.01,14.21)	9	5.75 (3.03,16.85)

Significant differences from the control group are denoted ** ($P<0.01$), and * ($P<0.05$), and between the tuberculoid and lepromatous groups are denoted ## ($P<0.01$).

Table 4.9: MHCII staining. Table of fluorescence intensity values (mean channel number).

Tissue	*MAb*	*Control*		*Lepromatous*		*Tuberculoid*	
		n=	*median (min,max)*	*n=*	*median (min,max)*	*n=*	*median (min,max)*
LPL	SW73.2	3	81.9 (58.8,92.6)	9	90.8 (73.7,99.6)	3	88.8 (81.9,105.2)
	VPM36	6	73.9 (60.2,82.6)	9	77.3 (58.4,83.2)	5	75.5 (73.7,89.6)
	VPM54	6	80.4 (62.9,97.2)	9	84.0 (56.1,89.0)	5	85.1 (67.6,101.3)
IEL	SW73.2	3	87.3 (82.8,100.6)	9	96.5 (71.8,100.9)	3	84.9 (84.4,94.2)
	VPM36	6	74.0 (64.8,98.3)	8	79.8 (61.6,96.0)	4	81.0 (72.8,82.6)
	VPM54	7	80.0 (65.8,103.1)	8	88.4 (58.9,103.9)	3	83.7 (79.3,85.7)
MLN	SW73.2	3	116.4 (105.7,134.0)	9	109.2 (84.7,121.7)	3	101.2 (99.2,131.4)
	VPM36	12	69.5 (57.2,122.1)	9	87.8 (75.0,100.6)*	5	81.3 (65.5,91.2)
	VPM54	12	90.8 (62.3,112.4)	9	89.0 (80.8,102.7)	5	85.3 (74.5,104.2)
PBL	SW73.2	4	110.9 (90.7,134.9)	13	111.0 (88.7,119.4)	5	104.7 (89.8,123.2)
	VPM36	16	68.4 (51.8,115.8)	12	82.0 (53.3,105.3)	6	75.9 (62.5,112.9)
	VPM54	16	77.2 (54.3,105.9)	11	101.5 (65.7,105.8)	5	82.7 (64.6,108.2)

* denotes significant difference from control group at P=0.03.

included in tables 4.3-4.6. No significant differences in the percentages of positively stained cells were found for any of the three MAbs in IEL, LPL or MLNL between the three groups of animals. However, significantly higher percentages of PBL from the lepromatous ($P<0.01$) and tuberculoid ($P<0.05$) groups were VPM36 positive (MHCII DQ) and a higher percentage of cells from the lepromatous group only were VPM54 positive when compared with the control group ($P<0.05$). No significant differences were detected in the percentages of positively stained cells between the two diseased groups. This was consistent with the finding of significantly higher percentages of VPM8 (sIg) positive cells in these groups. Fluorescence intensity values for MHCII staining are included in table 4.9. No significant differences were observed in the intensity of MHCII staining between groups, with the exception of the intensity of VPM36 staining in MLNL of the lepromatous group, which was significantly higher than the control group ($P=0.03$). However, this would not appear to be truly significant when viewed in the context of the range of values, and the lack of significant difference between the fluorescence intensity of the other MAbs for the MLNL of the three groups of animals.

4.4 DISCUSSION

IEL and LPL are distinct populations of cells in two distinct anatomical compartments. The description of the ileal IEL population herein broadly concurs with the findings of Gyorffy *et al.* (1992), for jejunal IEL in sheep: 54% were $CD8^+$, 6% $CD4^+$, although $\gamma\delta$ TCR^+ cells were slightly more frequent at 18%. Indeed, the IEL population described was not dissimilar to small-intestinal IEL in mice at 70% $CD8^+$, 11% $CD4^+$ and 31% $\gamma\delta$ TCR^+ (Camerini *et al.*, 1993), although in mice the high density of $\gamma\delta$ TCR^+ cells may be considered to be more highly significant due to the relative lack of these cells in the circulation. Waters *et al.* (1995), examined calves of 4-8 weeks of age and found that ileal IEL were approximately 6% $CD4^+$, 28% $CD8^+$,

and 23% γδ TCR$^+$. Only 6% expressed WC1 (T19), and 15% were B cells, which was thought to be due to the presence of continuous Peyer's patch in calves of this age. The first incubation of ileal mucosa in 2mM EDTA was performed in order to separate the epithelium from the underlying lamina propria by chelation (Nagi and Babiuk, 1987). Cells resulting from this digest were taken as an indicator corresponding to the cell population in the intestinal epithelium. In reality however, this sample was not a pure representation of the IEL population. On immunohistochemical analysis (chapter 3) there was a negligible number of CD4$^+$ cells present as IEL or in association with the epithelium, and Gorrell *et al.* (1988) found less than 1% of CD4$^+$ cells in the epithelium of 5-9 month old lambs on immunohistochemical staining. However, in the samples from the first EDTA digests, CD4$^+$ cells are present in significant numbers (median 10.3% in control sheep). This was probably a result of the scraping process to remove mucosa from the muscular layers of the ileum, or due to mechanical liberation of lamina propria cells during the first digest and removal of the epithelium. The tissue was from the outset in effect being disrupted from both superficial, epithelial and deep, mucosal facets. Gyorffy *et al.* (1992) and Waters *et al.* (1995) both identified 6% CD4$^+$ cells after EDTA isolation from the jejunal and ileal epithelia of lambs and calves respectively. In retrospect, since the outset of this study, an apparently better method with less scope for this error has been described, whereby lengths of intestine are not opened longitudinally, but everted and filled with buffer with the result that only the epithelial surface of the tissue is exposed to the EDTA/enzyme media (L. van Pinxteren, Moredun Institute, Edinburgh, personal communication). This could be anticipated to result in a more representative IEL population for analysis. The percentages of CD4$^+$ cells are even higher in the IEL fraction from diseased groups than from control animals, and this may be due to increased tissue friability in these animals, or higher density of lymphocytes in tuberculoid animals, and margination of lymphoid cells as noticed in lepromatous lesions on immunohistochemical staining. On addition of the

median percentages of the major T-cell subsets from the EDTA and enzyme digests (figure 4.4), it was noted that similar proportions of each subset were present between the control and lepromatous groups, but the tuberculoid group was characterised by predominance of the $CD4^+$ subset. The observations on IEL, nonetheless, show a predominance of $CD8^+$ cells in all groups, as was expected from immunostaining data (chapter 3), and concurred with the findings for jejunal IEL in lambs described by Gyorffy *et al.* (1992), who described 54% $CD8^+$ IEL. Gorrell *et al.* (1988) looked at immunohistochemical staining of epithelium and found less than 1% of $CD4^+$ cells in the epithelium of 5-9 month old lambs, and concluded that the IEL population was consequently $CD8^+$ in nature. The usage of the γδ TCR was not included in that study, although T19 was stained for, and the conclusion made that no $T19^+$ cells were found in the epithelium. Immunohistochemical staining found T19 expression to be rare in IEL (data not shown) and Mackay *et al.*, (1989) reported low numbers of $T19^+$ cells in the intestinal epithelium but that these cells were present in higher numbers in the LP. Waters *et al.* (1995) found that almost all γδ TCR^+ PBL co-expressed WC1, but only one third of γδ TCR^+ IEL in the ileum did. In this study, $T19^+$ cells are present in lower numbers than γδ TCR^+ cells in the epithelium. In mice, CD8 expression in the epithelium is mostly of the homodimeric (α/α) form, but in rats and humans these cells represent lower percentages of IEL (Guy-Grand and Vassalli, 1993). This phenotypic difference was not assessed in this study, as the individual glycoprotein chain specificities of MAb recognising ovine CD8 have not, to the writer's knowledge, been defined. Co-expression of CD8 and γδ TCR in IEL was not examined in this study, although Gyorffy and colleagues suggested that 4.3% of jejunal IEL were double-positive (which represented 24% of γδ TCR^+ IEL). Higher percentages of $CD4^+$ cells were present in the EDTA digests from tuberculoid cases than control animals and lepromatous cases. This was probably due to the higher densities of $CD4^+$ cells in the LP of tuberculoid cases, as was seen on immunohistochemical staining, and indeed in LPL isolated by enzyme digestion (table

4.4). Consequently the percentages of other cell subsets ($CD8^+$ and $\gamma\delta$ TCR^+) may be artificially low. The EDTA digest may be an indicator of the superficial cells of the lamina propria instead of a pure IEL population.

The findings for the percentages of lamina propria cells (table 4.5) to an extent reflected the immunohistochemistry findings. $CD4^+$ were the main subset present in LPL. Gyorffy *et al.* (1992) looked at the LPL of 6 lambs (8-12 weeks old) in the course of a study on $\gamma\delta$ TCR usage in sheep IEL, and described $37 \pm 4\%$ $CD4^+$ cells, $20 \pm 2\%$ $CD8^+$ and $10 \pm 2\%$ of $\gamma\delta$ TCR^+ cells. The percentage of $\gamma\delta$ TCR^+ cells was found to be lower in this study, and is likely to be due to the generally decreased percentages of $\gamma\delta$ TCR^+ lymphocytes in adult sheep compared with lambs (Hein and Mackay, 1991). The median percentage of $CD4^+$ cells was higher in the tuberculoid than the control group, and the control higher than the lepromatous group, although these differences were not statistically significant. Perhaps as a consequence, the percentage of $CD8^+$ cells was lower in the tuberculoid than the lepromatous group. The increase in $\gamma\delta$ T cells in the lepromatous group was reflected in the significant increase in T19 positive cells compared with control and tuberculoid groups, although the corresponding increase that was seen was paralleled in $\gamma\delta$ TCR^+ cells, again it was not statistically significant. The percentage of cells staining positively for CD5 was significantly higher in the tuberculoid than the lepromatous group. The pattern of CD5 staining is highly comparable with that of CD4 staining which may suggest that the specificity for this batch of antibody was not as it was initially believed to be. Similar patterns have been noticed in the data of other workers in this Faculty who have used the ST1 MAb (Begara *et al.*, 1995; Luján *et al.*, 1993). The higher percentage of $CD5^+$ cells in the tuberculoid group than the lepromatous group may therefore be a reflection of the increase in $CD4^+$ cells. Loss of CD5 is an indicator of lymphocyte activation in sheep lymphocytes (Hopkins and Dutia, 1990), and so an increase in the

percentage of $CD5^+$ cells, implying low levels of activation in tuberculoid cases, may not be expected.

The MLN of infected sheep were often grossly larger than those of control sheep and were the most frequent secondary site of infection (chapter 2). In a comparison of MLN and haemal nodes, Thorp *et al.* (1991) isolated MLNL from Merino ewes approximately 1 year of age, analysed the percentages of positively stained cells by flow cytometry, and described mean percentages of $CD4(SBUT4)^+$ 29±3%, $CD8(SBUT8)^+$ 24±4%, $T19^+$ 2±1%, and sIg 29±4%. The percentages of $CD4^+$ and $T19^+$ cells were comparable in this study, although the percentage of sIg^+ cells described here are higher, and $CD8^+$ percentages lower. Little variation was seen in MLNL populations between control and infected animals. The only significant differences to be noted between diseased and control tissues were higher percentages of both $\gamma\delta TCR^+$ and $T19^+$ cells in MLN of cases with lepromatous lesions of the ileum, which would appear to be consistent with the finding that these cells are present in higher percentages in the ileum of lepromatous cases. No other statistically significant differences were found between the MLNL of the three groups.

Phenotypic analysis of sheep PBL has been performed previously by a number of authors using different isolation procedures, and with variable findings. Maddox *et al.* (1985), characterised ovine CD4 and CD8 antigens using the MAbs SBUT4 and SBUT8 and found that the MAbs stained 20% (16-25) and 12% (6-14) of PBL respectively, and that 42% (23-57) of PBL expressed sIg. Mackay *et al.* (1989) found that PBL of 8-12 months old sheep comprised $CD4^+$ 20% (15-24) $CD8^+$ 11% (8 -15) $\gamma\delta$ TCR^+ 22% (15-30) and $T19^+$ 20% (14-28). Thorp *et al.* (1991), employed MAbs SBUT4 and SBUT8 in flow cytometric analysis of sheep PBL and described 14±6% $SBUT4^+$, 9±4% $SBUT8^+$, 25±4% $T19^+$ and 32±4% sIg^+ cells. Smith *et al* . (1994), looked at three blood samples from 12 sheep at two monthly intervals over a period of

six months starting at twelve months of age. No significant difference in the population was noted over the time course and so results were pooled. Mean percentages and ranges were $CD4^+$ 14.1% (8-22) $CD8^+$ 12% (4-22) $\gamma\delta$ TCR^+ 36.6% (22-68), and B cells 29.6% (11-50). The latter three workers used RBC lysis methods for the isolation of PBL whereas in this study Lymphoprep centrifugation was chosen as the method for isolation of PBL, and purification of cells isolated from all other tissues, and as such cells from all tissues were subjected to a standard, directly comparable procedure. It is possible, however, that Lymphoprep purification may selectively enrich or deplete certain cell subsets. The relatively low lymphocyte percentage figures found in this study were investigated by staining with MAb VPM18 (anti-CD45, Leukocyte common antigen) as a positive control in order to verify that no RBCs were contaminating the gated lymphocyte population, and 99.5% cells within the lymphocyte gate were in fact CD45 positive (n=5, range 98.4-99.6%). Other leukocytes present in the lymphocyte gate may have depressed the percentages of positively stained cells, although the presence of granulocytes and monocytes would be unlikely due to exclusion on the basis of different FSC and SSC characteristics. It is noteworthy that a subset of ovine lymphocytes has been described which is $CD3^+$, but negative for CD4, CD8, T19 and $\gamma\delta$ TCR molecules, and Keating (1995) consistently found approximately 5% of such "null" cells in the PBL and afferent lymph of sheep. The same number of cells was used in each tube for each animal and tissue, however the concentrations of $CD4^+$ and $CD8^+$ cells may have been much higher in PBL than in other tissues. The CD4:CD8 ratios for the PBL of control animals were not as expected, and other workers have in the main described percentages in the reverse proportions from those of the control animals described here. FACS analysis percentage tables (appendices 4.2-4.4) show that only 4 of 17 control-animal PBL were found to have higher percentages of $CD4^+$ than $CD8^+$ cells. Initially a technical fault in the MAb staining of the cells was suspected, perhaps over diluted or decaying SBUT4, or non-specific staining by SBUT8, however the same

batches and dilutions were used for all other tissues from the same sheep, and no inversion of the expected ratios was seen in cells from these tissues. The relative T lymphocyte and $CD4^+$ concentrations may have been higher in control PBL than in other tissues, and as a consequence SBUT4 may have been in effect insufficiently concentrated. However, staining with the ST1 MAb consistently paralleled staining with SBUT4 in percentage of positively stained cells even in PBL, and was in the form of saturated hybridoma supernatant, which would tend to validate the appropriateness of the SBUT4 ascitic fluid titration, and indicate that the apparently low percentages were not the result of a problem with this MAb. Surface Ig may not be the best antigen for the quantitation of B cells, since it may be only more mature B cells which express these, and so a number of B cells may not have stained positively and depressed total lymphocyte numbers. In addition, plasma cells may not express sIg. Some B cells may also scatter light more due to increased cytoplasm and higher density of cytoplasmic cell organelles responsible for immunoglobulin production and a consequently lowered nucleus-cytoplasm ratio, and so be outwith the lymphocyte box selected (Smith *et al.*, 1994). Nevertheless, percentages of circulating B cells were found to be significantly higher in the PBL of both groups of diseased animals compared with normal controls, and would suggest a systemic antibody type response to infection with *Map*. The relative increase in the percentages of B cells may be responsible for the decrease seen in $CD8^+$ and $\gamma\delta$ TCR^+ T lymphocyte percentages, or this may possibly be due to recruitment of cells of these phenotypes into paratuberculosis lesions.

The phenotypes of peripheral blood T-lymphocyte subsets have been studied in human patients with ulcerative colitis (UC) and Crohn's disease (CD) (Selby and Jewell, 1983) and no significant differences were found in the proportions of T-cell subsets or CD4:CD8 ratios between patients and normal controls. The absolute numbers of circulating T cells were reduced, however, in patients with these

inflammatory bowel diseases (IBD), and the number of $CD4^+$ cells reduced in UC, and both $CD4^+$ and $CD8^+$ cell numbers reduced in the active form of CD, compared with normal subjects. A similar study was performed by Giacomelli *et al.* (1994), and no significant differences found in total ($CD3^+$) and $CD4^+$ circulating T lymphocytes, but a significant reduction in the percentage of $CD8^+$ lymphocytes in patients with UC and CD. Interestingly, the percentage of $\gamma\delta$ TCR^+ PBL was found to be significantly and markedly increased in patients with active CD, and the authors suggest that this may be related to the granulomatous nature of CD lesions.

MHCII staining of LPL revealed no significant differences between control and diseased animals, however this assessment was of single fluorescence staining of all cells and not double-staining of T cell subsets. Further double-staining experiments would be necessary in order to assess T-cell activation in the lesions of paratuberculosis. Activation of gut T cells of normal sheep was assessed by Gyorffy *et al.* (1992) by double staining for MHCII and was found to be high in both IEL and LPL compartments, with greater than 80% of all T cells expressing MHCII, in contrast with less than 40% of MLNL and less than 20% of T cells from peripheral (prescapular) LN. LPL of non-human primates have also been assessed, and higher percentages were found to be activated than MLNL and PBL by comparison, on the basis of CD25 and MHCII expression (Zeitz *et al.*, 1991).

The proportions of the T-cell subsets within the lymphocyte populations isolated from the ileum of infected animals and normal control sheep are broadly comparable with those described *in situ* by immunoperoxidase staining. The FACS analysis findings suggest that paratuberculosis cases with tuberculoid lesions have higher proportions of $CD4^+$ cells, and that lepromatous cases are associated with higher percentages of $\gamma\delta$ T cells, both in the ileal tissues, and in the MLN. The most striking finding for the PBL of the infected animals was that both groups had significantly

higher percentages of sIg^+ B cells, and this was reflected in the corresponding increase in MHCII positive cells.

CHAPTER FIVE

SERUM ANTIBODY LEVELS AND LYMPHOCYTE PROLIFERATIVE RESPONSES IN OVINE PARATUBERCULOSIS

5.1 INTRODUCTION

Protection against mycobacterial disease is dependent on T-cell mediated immunity, with responses being transferable from immunocompetent animals to naive recipients (Orme and Collins, 1983). Furthermore, immunity can be impaired or abrogated by depletion of T-cell subsets, either mediated by antibody or using gene-knockout technology. Likewise, T-cell dependent immunity has been shown to be a feature of paratuberculosis, and in the murine model T cells are necessary for a protective response to *Map* infection, as athymic, nude mice succumb to infection which results in intestinal multiplication of the organisms and subsequent death (Hamilton *et al.*, 1989). $CD4^+$ cells are thought to be the subset necessary for protective responses to intracellular bacterial pathogens, and in addition to helper-type roles, the ability to lyse target cells which had been primed with mycobacterial antigen has also been demonstrated (Boom *et al.*, 1991). The same ability has been described for $\gamma\delta$ TCR^+ T cells (Munk *et al.*, 1990), and $CD8^+$ cells are known to respond to mycobacterial antigen in a MHC class I restricted manner (Flynn *et al.,* 1992). Hence, all three major phenotypic subpopulations of T lymphocytes are involved in mediating protection against mycobacterial infections. However the kinetics of the immune response in ruminant cases of natural infection still requires clarification.

The aim of this study was to assess the proliferative responses to *Map* antigen by lymphocytes both of control and infected animals, and of peripheral blood and local

tissue origin. Previous studies have reported diminished lymphocyte proliferative capacity in PBL isolated from bovine clinical paratuberculosis cases (Kreeger and Snider, 1992 ; Kreeger *et al.*, 1992). The use of lymphocyte stimulation assays (LSA) to test cell-mediated immunity (CMI) in *Map* infection is well established, with this technique having been described previously by several workers (Alhaji *et al.*, 1974; Buergelt *et al.*, 1978; De Lisle and Duncan, 1981; Gilot *et al.*, 1992; Milner *et al.*, 1981). Lymphocyte stimulation assays are based on the incorporation of radio-labelled thymidine into the replicating DNA of cells proliferating in response to mycobacterial antigen. This technique is not normally used in a diagnostic capacity. Johnin purified protein derivative (JPPD) is the antigen preparation which has been most extensively used for this purpose, and is perhaps more correctly considered as a sensitin, a heterogeneous mixture of substances which induce non-specific inflammatory reactions, and of antigens which have extensive cross reactivity throughout the genus *Mycobacterium*. Hence PPD is not an optimal preparation for use as a test antigen due to the lack of genuine specificity to *Map* (Gilot and Cocito, 1993). Cross reaction with mycobacterial antigens in particular, and with those of the CMN group is likely, and as grazing animals sheep may well be naturally exposed to high numbers of these antigens. *Map*-specific antigens would be more desirable for use in this type of assay, and indeed several *Map* antigens have been identified (Gilot *et al.*, 1992; Burrells *et al.*, 1995; and reviewed in Cocito *et al.*, 1994). Such antigens may at some stage in the future be more widely available, and more easily produced in such quantities as to make them available to studies such as this.

Responses to mycobacterial infection, and to intracellular pathogens in general, are T cell mediated and, in the main, MHC restricted. Presentation of antigen requires antigen presenting cells (APC) and is associated with processing of exogenous peptide in association with MHCII. PPD is a crude antigen preparation, which by definition is mostly peptide in nature, and is likely to be processed via this pathway. Recently, γδ

TCR^+ cells have been shown to be capable of recognising non-peptide antigen in association with non-classical molecules, for example by molecules of the CD1 lineage, (lipid/carbohydrate antigens) and whole organisms (reviewed by Kaufmann, 1995, and Lanier, 1995). A high repertoire of γδ TCR^+ epitopes have been described in ruminants and sheep compared with other non-ruminant species (Walker *et al.*, 1994), and γδ TCR^+ T cells are considered to be a highly significant T-cell subset in this species (Hein and Mackay, 1991). Traditionally, most studies have concentrated on PBMC responses to antigen, perhaps due to the ease of isolation of these cells, but in this case it was considered important to examine in addition the responses of cells of the local immune system. GALT cells recirculate through MLN and into the lymphatic system, returning via the thoracic duct to the bloodstream, and subsequently re-home to GALT (Mackay *et al.,* 1990). This population is functionally and to some extent antigenically different from circulating PBL, and as such examination of their proliferative responses may provide information on the pathogenesis of *Map* infection.

Differential responses of PBL and LPL have been reported, for example poor proliferation of LPL has been described in mixed lymphocyte reaction assays, and this cell population has been considered to comprise a high proportion of antigen specific memory cells with a high degree of activation (Elson *et al.*, 1982; Zeitz *et al.*, 1991; James and Zeitz, 1994). The implication is that gut-specific cells react differently in response to antigen compared with PBL and investigation and comparison of the responses of lymphocytes isolated from the three different sites (chapter 4) may provide information on the type and magnitude of this response. Likewise, proliferative responses to *Map* antigen will be related to the histopathology observed in the gut lesions (chapter 2).

Humoral responses are thought to be of lesser importance in mycobacterial disease, and are unlikely to play a significant role in the elimination of infection. An

inverse relationship between CMI and humoral response has been proposed, with CMI dominant in the early stages of infection, and high serum antibody concomitant with waning CMI as the disease progresses (Bendixen, 1978; Chiodini *et al.*, 1984). Measurement of CMI versus humoral responses will be examined.

5.2 MATERIALS AND METHODS

5.2.1 Sheep

Nineteen adult ewes clinically affected with paratuberculosis and sixteen normal control sheep were used in this study. Animals were subjected to full necropsy examination (chapter 2) and on the basis of histopathological findings were classified as described into lepromatous (n=11) and tuberculoid (n=8) groups.

5.2.2 Collection of tissues and lymphocyte isolation

Blood, ileum and MLN were collected as previously described (chapter 4). PBL were isolated by Lymphoprep centrifugation (section 4.2.7). MLNL were isolated by cutting MLN into small pieces, and rubbing gently on small-gauge wire gauze immersed in RPMI-FCS media in a Petri dish, and the resultant cell suspension collected by pipette. MLNL were then purified by Lymphoprep centrifugation in RPMI-FCS. Ileal LPL were isolated and purified as described earlier (section 4.2 6). All cell suspensions were washed in RPMI-FCS by centrifugation at 650 x g, and resuspended in RPMI-FCS at a concentration of 1 x 10^6 ml^{-1} prior to aliquoting into assays.

5.2.3 Antibody ELISA

In addition to AGID tests for *Map* specific antibody, plasma *Map* specific antibody levels were assessed by *M. phlei*-absorbed ELISA which provided a quantitative method for detection of *Map* specific antibody. A plasma sample from blood samples was stored at -20°C until use. ELISA testing was performed by Dr C. Burrells at the Moredun Research Institute. Plasma was incubated with *M. phlei* to provide absorption of non-specific anti mycobacterial antibodies (Milner *et al.*, 1990). 40 mg of *M. phlei* culture was added to 1 ml of a 1:100 dilution of plasma in PBS containing 0.05% polyoxyethylene sorbitan monolaurate (Tween 20) (PBS-tween) and incubated overnight at 4°C. Prior to addition to the ELISA plate, *M. phlei* was sedimented out of suspension by centrifugaton at 2000 x g for 10 minutes. ELISA plates (M129A - Dynatech Laboratories, Billingshurst, W. Sussex) were prepared by coating each well with 100 µl coating buffer (carbonate/bicarbonate) containing 10 µg ml^{-1} *Map* lysate (Burrells *et al.*, 1995) by incubation overnight at 4°C. Negative control wells were produced by adding coating buffer only, without antigen, to each alternate row. Plates were then washed three times in PBS-tween, and non-specific binding blocked by incubating each well with 100 µl of 10% milk powder ('Marvel', Premier Brands UK Ltd., Adbaston, Staffs.) in coating buffer for 1 hour at room temperature. Following a further wash, 100 µl of *M. phlei*-absorbed plasma sample was added to duplicate coated and uncoated, negative-control wells. A series of ten doubling dilutions of reference positive serum and standard positive and negative control plasma samples was added to the first test plate of each batch. Plates were incubated for 1 hour at room temperature (RT), and thereafter washed before 100 µl of horseradish peroxidase conjugated donkey anti-sheep IgG (Scottish Antibody Production Unit, Law Hospital, Carluke, Lanarkshire) diluted 1:100 in 10% 'Marvel' in PBS-tween was added. Plates were then incubated for 1 hour at RT, washed and 100 µl of OPD (orthophenylenediamine dihydrochloride, Sigma.) substrate added to

each well. Plates were incubated in the dark at RT for 30 minutes before the development reaction was stopped by addition of 50 µl of 2.5M H_2SO_4. The optical density (OD) of the wells was determined by reading at a wavelength of 490nm on a Dynatech MR 5000 ELISA plate reader (Dynatech Laboratories). Results were calculated using the Dynatech Reader Manager software, and the first dilution of the 10 reference standard serum samples given a value of 100 ELISA Antibody Units (EAU) and a reference standard curve constructed by plotting the OD of the standard dilutions against their Log EAU values. Mean OD values were calculated for each duplicate test sample and these corrected by subtraction of the mean OD of the two paired negative control wells. The EAU for each corrected OD value was computed by reference to the standard curve.

5.2.4 Lymphocyte stimulation assay

A lymphocyte stimulation assay (LSA) using Johnin PPD antigen was performed in order to make an assessment of cell mediated immune responses to *Map* antigen. With the aim of consistency, LSA assays were in the main performed in the Moredun Institute by Dr C. Burrells, and the assay protocol has been previously published (Burrells and Wells, 1977; Burrells *et al.,* 1995). In brief, 200 µl of cell suspension (1×10^6 ml^{-1}) were aliquoted in triplicate into sterile 96 multiwell flat-bottomed microwell cell-culture plates (Nunc, Gibco BRL Life Technologies). For each assay, triplicate wells were used for negative control, mitogen positive control, and paratuberculosis antigen. The negative control was 20 µl of media (RPMI-FCS). The mitogen positive control for lymphocyte stimulation was 20 µl of Concavalin A (Con A) (ICN Biochemicals, High Wycombe, Bucks) diluted in RPMI-FCS to provide a final concentration in the test of 7.5 µg ml^{-1}. The antigen used to test lymphocyte response was 20 µl of JPPD ('Johnin', Central Veterinary Laboratory,

Weybridge, Surrey), and this was diluted in RPMI-FCS to provide a final test concentration of 6.25 μg ml^{-1}. To each well, 200 μl of cell suspension was added, equivalent to 2 x 10^5 cells. Plates were then covered and incubated in a humid tissue culture incubator at 37°C for five days, with 5% CO_2 supplementing atmospheric air. On day four, sixteen hours prior to the end of incubation, cells were "pulsed" by addition of tritiated (methyl H^3) thymidine of activity equivalent to 1 μCi (micro-Curie) (5 Ci $mmol^{-1}$) (Amersham International, Little Chalfont, Bucks), diluted in 20 μl RPMI-FCS, and incubated for sixteen hours (overnight). Following culture, cell suspensions were harvested and radioactive count determined using a direct beta counter ('Matrix 96', Packard Instruments, Reading, Berks). From the mean counts per minute (cpm) output, stimulation indices (SI) were calculated by division of the mean cpm of antigen stimulated cultures, by the mean cpm of the unstimulated, media control cultures. Single aberrant counts, when they occurred, were excluded from the mean cpm figure calculation, and may have been the result of contamination of the culture well.

5.2.5 Statistics

Data were plotted in histogram form, and were found to be not consistently normally distributed. Data were transformed logarithmically both to facilitate direct comparison between different units of measurement, and to provide a distribution more consistent with the normal distribution, but even following transformation the data were still apparently skewed, although the distribution was similar for each group. Consequently, non-parametric statistical methods were employed in the data analysis. The Mann-Whitney confidence interval and test was chosen to compare the median values between two groups of data, and the Kruskall Wallis test for three groups. Correlations were examined between data sets using the Spearman's Rank

Correlation test, and significance levels for the resultant correlation coefficients determined using critical value tables. Geometric mean values with 95% confidence intervals are given to provide an indication of the expected mean values for SI and EAU, and can be considered in the case of skewed data to return a truer value than the arithmetic mean in this situation.

5.3 RESULTS

5.3.1 Antibody

Serum antibody status of animals was assessed initially using the AGID test (Sherman *et al.*, 1984), and testing was performed by the Scottish Agricultural Colleges' Veterinary Investigation Laboratories, Bush Estate, Midlothian. All control animals gave a negative test result. Ten of the eleven animals which comprised the lepromatous group were found to be positive for mycobacterial antibody, whereas only two of the eight animals of the tuberculoid group were determined to be positive by this method.

EAU values for individual animals are shown in figures 5.1 and 5.2. Median and geometric mean values are shown in tables 5.1 and 5.2 respectively, and are represented graphically in figure 5.3.

All control sheep had previously provided a negative AGID test result. On ELISA testing, the EAU levels of the control group were of low magnitude, although 6 of the 16 control animals had EAU levels which were considered to be positive at the assigned cut-off value of 50 EAU (53.6-65.8 EAU). When compared with the EAU

Figure 5.1: Serum EAU and PBL LSA values for sheep of the control group.

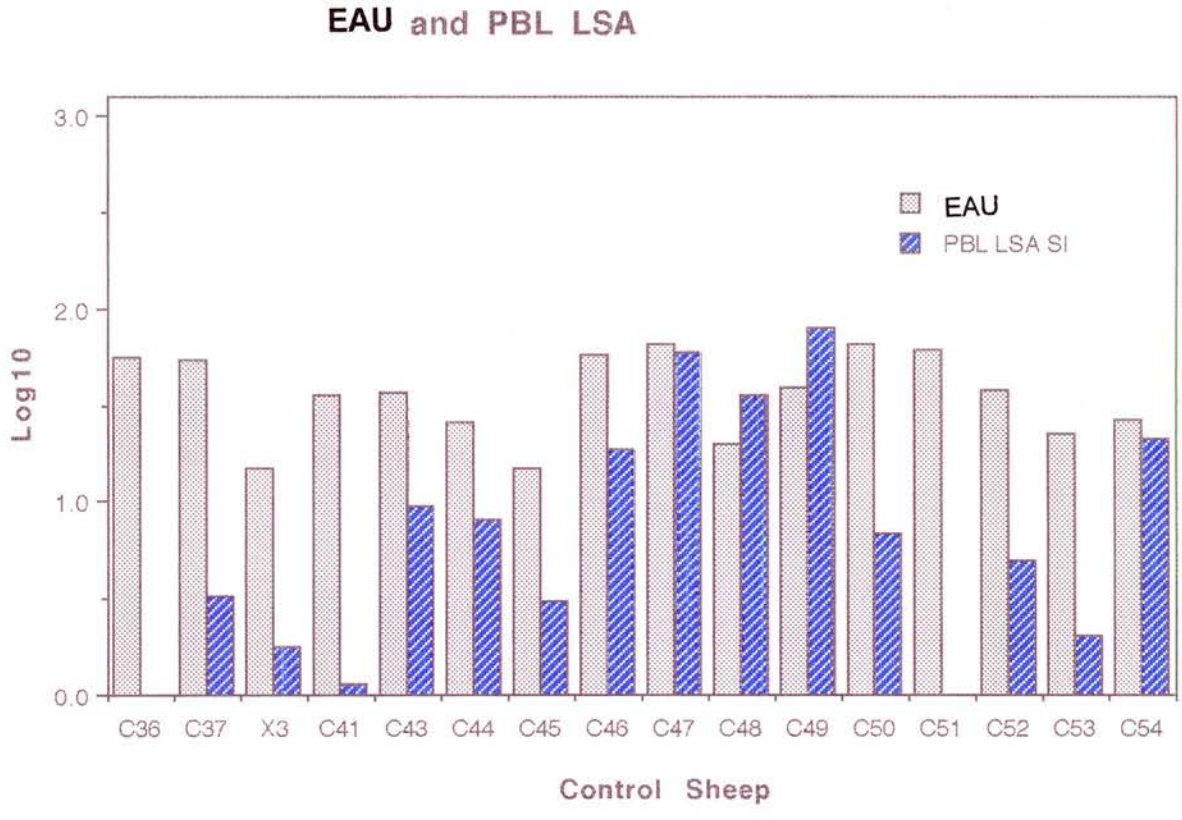


Figure 5.2: Serum EAU and PBL LSA values for sheep of the lepromatous and tuberculoid groups.

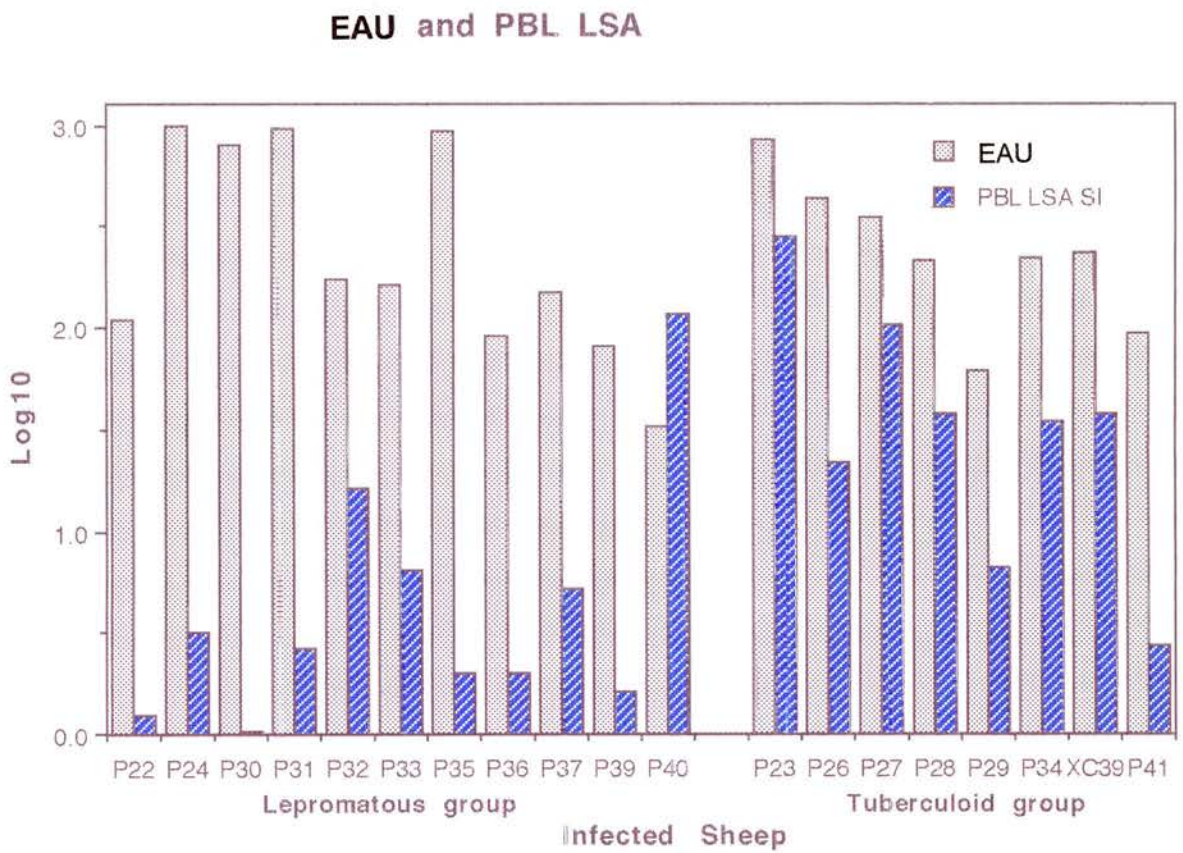


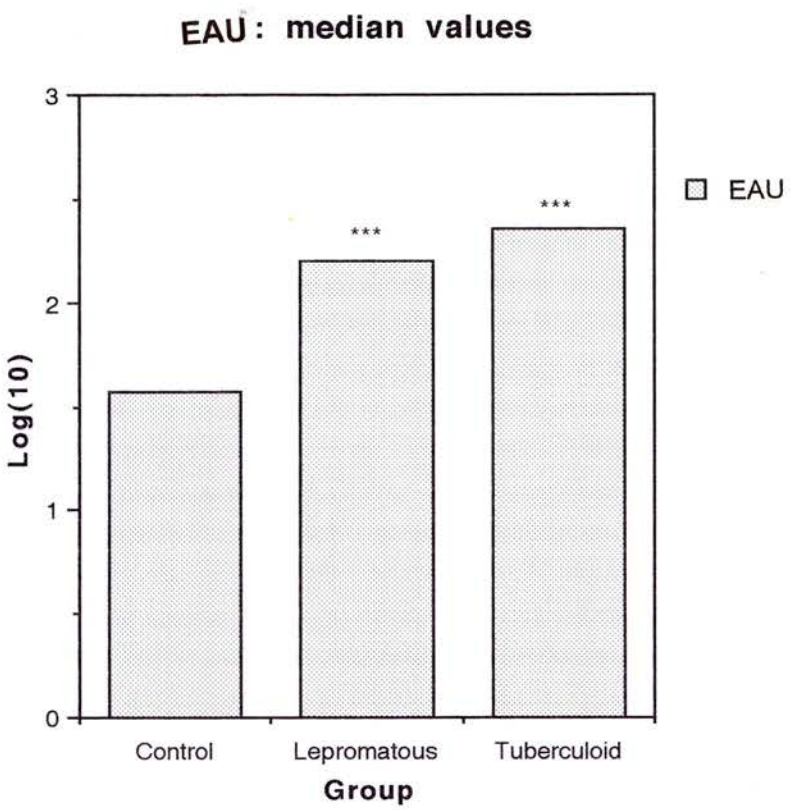
Table 5.1 EAU and LSA SI values (median and range). Significant differences from the control group are denoted *** (P<0.001) and * (P<0.05), and between tuberculoid and lepromatous groups are denoted ## (P<0.01) and # (P<0.05).

	Control	*Lepromatous*	*Tuberculoid*
EAU	37.4 (14.9,65.8)	161.1 (32.5,958.9)***	228.3 (60.8,851.7)***
PBL LSA SI	5.9 (0.6,78.1)	2.7 (1.7,118.2)	36.2 (2.8,282.9)*##
MLNL LSA SI	2.5 (0.6,21.8)	1.5 (0.9,9.8)	18.5 (1.4,115.9)*#
LPL LSA SI	1.4 (0.4,6.6)	1.1 (0.8,104.0)	6.2 (0.8,37.3)*

Table 5.2: EAU and LSA SI values for each group of animals: Geometric mean and 95% CI.

	Control	*Lepromatous*	*Tuberculoid*
EAU	35.5 (27.0,46.6)	224.9 (100.5,504.7)	231.7 (115.4,465.6)
PBL LSA SI	6.12 (2.78,13.52)	4.03 (1.60,10.16)	29.58 (8.79,99.54)
MLNL LSA SI	3.39 (1.71,6.73)	2.08 (1.19,3.6)	14.42 (3.48,59.84)
LPL LSA SI	1.32 (0.84,2.06)	1.73 (0.68,4.42)	5.32 (1.17,24.21)

Figure 5.3: Median EAU values for all three groups of animals. Significant difference between infected and control animals are denoted *** (P<0.001).



for both infected groups, the median of the control group (37.4 EAU) was found to be significantly lower than those of both infected groups (both $P<0.001$).

Ten of the eleven sheep of the lepromatous group were positive for mycobacterial antibody on AGID test (with the exception of sheep P37). One animal in this group was negative on ELISA test (P40, 32.5 EAU) with the other animals ranging between 80-1000 EAU (median 161.1 EAU). Only 2 sheep of the tuberculoid group provided a positive AGID test result (P23 and P34), whereas all 8 sheep in this group had positive antibody levels detected by the ELISA method (median 228.3, range 61.0-851.2 EAU). No significant difference was apparent between the median values of both infected groups ($P=0.84$).

5.3.2 LSA

LSA stimulation indices are listed in tables 5.1 and 5.2, and shown for PBL isolated from individual animals in figures 5.1 and 5.2, and for MLNL and LPL in figures 5.4. and 5.5. The median lymphocyte proliferative responses (figure 5.6) were found to be of the highest magnitude in PBL, which were then followed by MLNL, with LPL showing the lowest levels of proliferation. A factor of two was observed between the magnitude of PBL and MLNL stimulation indices, with the level of LPL responses being very much lower (figure 5.6). In addition, a similar pattern was evident within the responses for each group of animals (identified on the basis of histological lesions), with PBL showing greater incorporation of tritiated thymidine than MLNL, and in turn than LPL, for tuberculoid, lepromatous and control groups.

Figure 5.4: LSA SI values for MLNL and LPL isolated from sheep of the control group. ND: not determined.

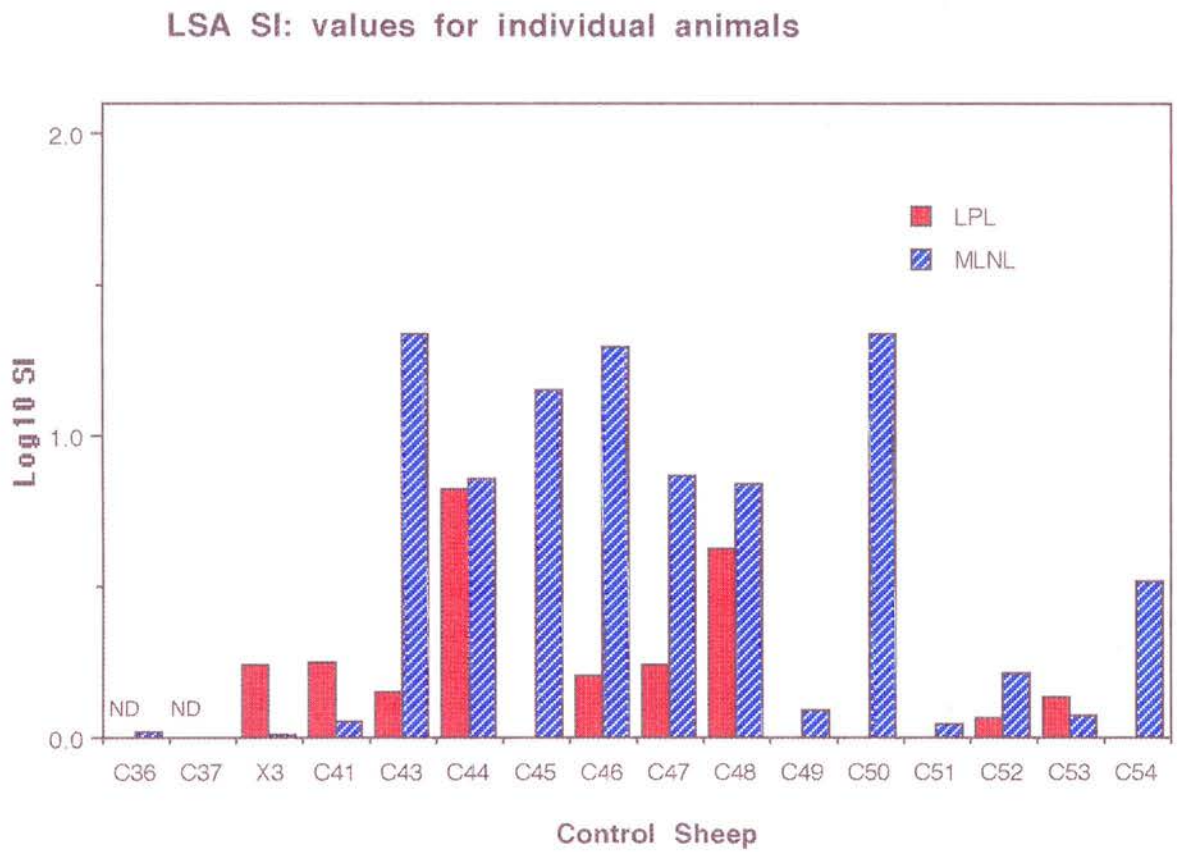


Figure 5.5: LSA SI values for MLNL and LPL isolated from sheep of the lepromatous and tuberculoid groups. ND: not determined.

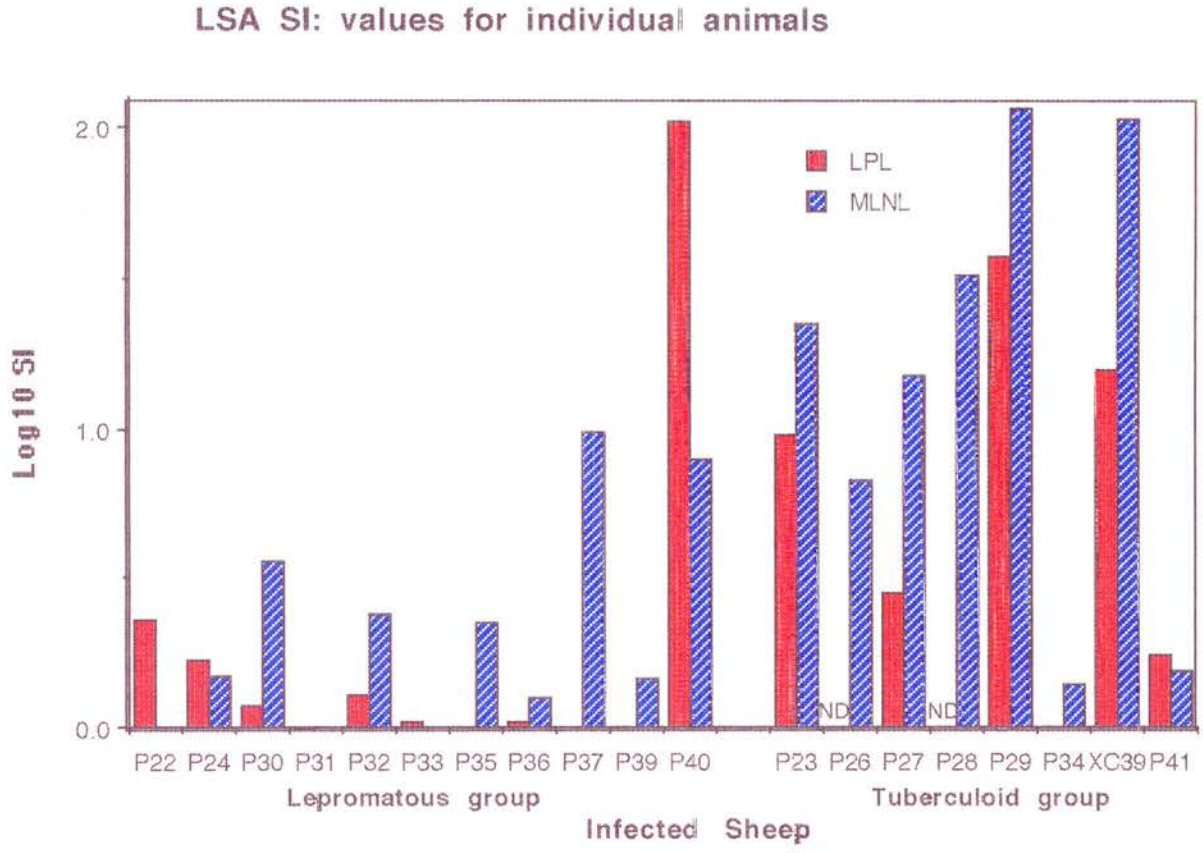
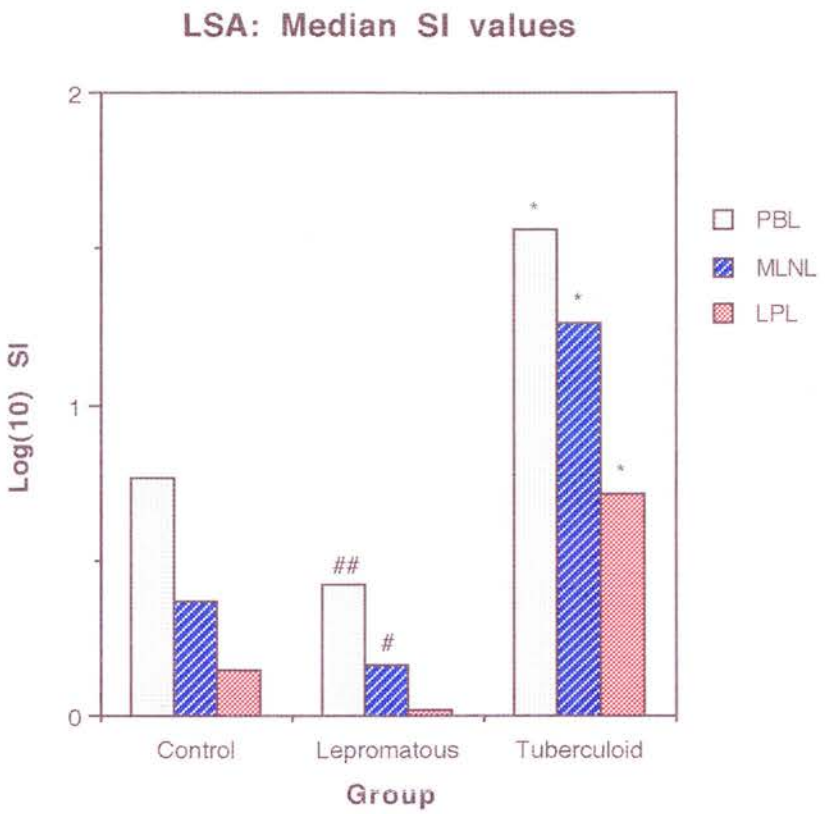


Figure 5.6: Median LSA SI values for PBL, MLNL and LPL isolated from control and infected sheep. Significant differences from the control group are denoted * (P<0.05), and between infected groups # (P<0.05) and ## (P<0.01).



5.3.3 PBL LSA

Five of the control sheep showed no proliferative response by PBL to JPPD (SI ≤ 3) whereas the remaining eleven had responses ranging from SI 3.1-78.1 (median SI of the whole group was 5.9).

PBL from five animals of the lepromatous group gave positive proliferative responses, with one sheep (P40) giving a strong proliferative response (SI 118.2). However, the responses of the other animals in this group were slight in comparison (3.2-16.2). No significant difference was found between the medians of the lepromatous and control groups. Seven animals of the tuberculoid group had a positive PBL LSA response to JPPD (SI 6.6-282.9) with only one animal (P41) considered negative with an SI of 2.8. The proliferative responses of this group were found to be significantly higher than both the control ($P<0.05$) and lepromatous ($P<0.01$) groups.

5.3.4 MLNL LSA

Eight of the sixteen animals of the control group showed proliferative MLNL responses to JPPD (Positive SI range 3.3-21.8; Median of all animals 2.5).

MLNL LSA responses in three sheep of the lepromatous group were positive (SI 3.6-9.8) while the remaining eight were all considered negative. No significant difference was found between this and the control groups. MLNL isolated from animals with tuberculoid lesions had significantly higher proliferative responses than the control ($P<0.05$) and lepromatous ($P<0.05$) groups, with six of eight animals having a positive proliferative response (median 18.5).

5.3.5 LPL LSA

LPL were isolated from fourteen control animals, and positive proliferative responses of low magnitude were found in two animals in this group (SI 4.2 and 6.6).

LPL from only one animal of the eleven tested from the lepromatous group gave a positive result (SI 104.0), and this was sheep P40 which also had high PBL and MLNL responses. The other animals in this group were considered negative, and no significant difference was noted between the medians of the lepromatous and control groups.

LPL were assayed from six animals of the tuberculoid group, and three animals gave positive SI of 9.6, 15.7 and 37.3 (group median 6.2). This was significantly higher than median SI of LPL from control animals ($P<0.05$).

5.3.6 Summary

No significant differences in the LSA SI for antigen specific proliferation to JPPD were detectable between the control and lepromatous groups for lymphocytes from any of the tissues studied, although median values were consistently lower in the lepromatous group compared with the control group. However, in the case of tuberculoid animals, all tissue lymphocytes had significantly higher proliferative responses than equivalent cells isolated from the control group ($P<0.05$). Between both infected groups, PBL from tuberculoid animals had very significantly higher median SI values than those of the lepromatous group ($P<0.01$), and those of the MLNL were likewise significantly higher than lepromatous cases ($P<0.05$).

5.3.7 Correlations

Significant positive correlations were found only between PBL and MLNL LSA SI for the control group (modest correlation, $r=0.565$, $P<0.05$) and between LPL and MLNL for the tuberculoid group (very strong correlation, $r=0.995$, $P<0.05$).

5.4 DISCUSSION

Antibody levels were examined in this study both as a means of confirmation of the initial clinical diagnosis, and as an indicator of the animals' humoral immunity. The detection of the presence of *Map* specific antibody is a recognised diagnostic test for paratuberculosis, and is normally assessed by AGID, ELISA or complement fixation (CFT) serological tests. The sensitivity of such serological tests as an indicator of infection is not particularly high, and the search for a highly sensitive assay continues to be a priority in paratuberculosis research. Antibody is considered to be of value in diagnosing only animals in the later stages of paratuberculosis, with low levels of antibody being produced in the earlier, subclinical stages. As a result, a high number of false negatives are described with resultant low sensitivity in animals in the earlier stages of infection, which are in general not able to be diagnosed by this method (reviewed by Kreeger, 1991). Animals in the early stages of disease are in general thought to have dominant cell-mediated immunity, with the switch to antibody production occurring as the infection persists and progresses.

Chiodini *et al.*, (1984) proposed that paratuberculosis is associated with high levels of antibody in the later, terminal stages of advanced infection and is similarly associated with a waning CMI response in these animals. Similar observations have been made in a review of immune reactivity in bovine paratuberculosis by Bendixen (1978), but were based not on *in-vitro* lymphocyte assays, but on the *in-vivo*

cutaneous DTH reaction to intradermal Johnin injection. This may have been a reflection of the relative prevalence of the lepromatous form of the disease in cattle, where fewer cases of the tuberculoid type lesions described in sheep appear to be found (Buergelt *et al.*, 1978). De Lisle and Duncan (1981) described consistent proliferative responses by lymphocytes isolated from 'minimally infected' animals, and these animals were also found to be negative for both serological and intra-dermal tests. 'Heavily infected' animals, however, showed considerable variation in their lymphocyte proliferative responses to antigen, with some animals being consistently unresponsive. Proliferative responses were also noted in the control animals in that study, particularly in a large percentage of non-infected controls from herds with a history of endemic paratuberculosis. This concurs with the observations in this study, and may suggest an exposed and resistant state in these animals.

The AGID test was employed for detection of ovine paratuberculosis in a study of sheep culled from paratuberculosis affected flocks, and low sensitivity was reported in the majority of animals which had limited, early type histological lesions (García-Marín, 1991). This was in agreement with the findings of Sherman *et al.*, (1990), who described low sensitivity in pre-clinical cases of bovine paratuberculosis, and in addition found a positive correlation between the presence of faecal AFB and a positive AGID test result. Pérez *et al.*, (1994), evaluated the AGID and IFNγ tests in experimentally infected sheep and found that animals gave a positive IFNγ test result 'immediately' at 50 days, and the first positive AGID test result at 120 days post-infection. In a similar study in the bovine, Yokimozo *et al.*, (1994), described positive LSA and IFNγ test results at 2-4 months, and positive antibody ELISA test results at between 8-10 months. One study in addition examined seroconversion in experimental infection by ELISA and suggested that this took place between 10 and 24 months post-infection (Milner, 1987).

In this study, no significant difference was noted in serum antibody levels measured by ELISA between both tuberculoid and lepromatous groups. However all animals were in the advanced stages of disease, and none fell into the category of early lesions, as described by Pérez *et al.* (1996) (lesions of types I and II by their classification). Antibody to *Map* was detectable in fewer of the tuberculoid group by AGID than by ELISA, and this is likely to be a reflection of the relative sensitivity of the tests (Dimareli-Malli *et al.*, 1991). Likewise, specificity of the AGID test may be expected to be lower as a result of the use of Johnin PPD as test-antigen, to which mycobacterial antibodies that are non-specific to *Map* may react. The use in the ELISA test of *M. phlei* absorption of non-specific anti-mycobacterial antibodies increases the specificity of this technique. As was discussed in chapter 2, however, a positive correlation was found to exist between the presence of high AFB burdens and serum antibody as detected by the AGID test. No correlation was found between ELISA antibody units and LSA SI, although the sensitivity of the ELISA test is high and correlation may have been noted if AGID results had been considered. However, AGID test results are binary observations in nature, and as such are qualitative rather than quantitative. Moreover, it should be noted that EAU cannot be considered a truly quantitative measurement of antibody concentration. A sample which was ascertained to have a score of 1000 EAU is unlikely to equate to double the antibody concentration of a sample of 500 EAU. For this reason, geometric mean values (and their confidence intervals) were provided as a more robust statistic than simple mean or median values, and in spite of the data being non-normally distributed, can be considered valid statistics for the expression of these results.

An inverse relationship is suggested between humoral and cell-mediated immunity, both by the existence of the Th1/Th2 paradigm, and from clinical observations in other mycobacterial diseases. However, in cases of progressive disease, increasing bacillary burden and increasing exposure to mycobacterial antigen

due to liberation of AFB in high numbers from decaying or lysed macrophages might be expected to result in increasing levels of serum antibody. Of interest in the results of individual animals was sheep P40 of the lepromatous group, which appeared to be anomalous in that it was found to be negative for antibody by ELISA (although initially positive by AGID), and had a high LSA SI value (and in addition was positive for IFNγ production - chapter 7). This would appear to be in conflict with the accepted hypothesis of low CMI and high humoral immunity for the lepromatous form of the disease.

Cell-mediated immune responses were assayed by using LSA as an indicator of antigen-specific proliferation and expansion by memory cells. Lymphocyte proliferation occurs in response to T-cell recognisable antigen presented in association with cell-surface molecules of APC (by both classical, MHC-restricted and non-classical pathways). In this assay, Johnin PPD was used as the test antigen preparation, but PPD is a relatively crude and uncharacterised antigen preparation, and cross-reactions are known to occur with other mycobacterial species, and with other bacteria within the CMN group of organisms (Chiodini *et al.*, 1984; Cocito *et al.*, 1994). Hence, an antigen with known *Map* specificity would be more desirable. Likewise, antigens used as reagents in immunoassays ought to possess T-cell-specific epitopes for CMI testing, and B-cell-specific epitopes if used for serological testing. Many of the control animals showed lymphocyte proliferative responses to PPD, suggesting the existence of antigenic cross reaction, and/or previous exposure to mycobacterial antigens. PPD may be more likely to be processed and presented via the MHCII pathway, and thus elicit a $CD4^+$ restricted response, or indeed a Th1 type response (Parronchi *et al.,* 1991). The nature of antigen in infection may dictate the development of specific type responses. A study of human leishmaniasis showed that Th1 and Th2 cells bear distinct T-cell receptors and thereby are likely to recognise distinct antigens (Uyemura *et al.,* 1993). In addition, differences in responses to live

versus killed bacteria, and whole organisms versus extracts have been previously noted (section 5.1) in terms of the T-cell subset responding, and thereby in the effector mechanisms.

Different levels of response were noted for lymphocytes isolated from each tissue. On comparison of responses according to tissue of origin, the highest proliferative responses (as measured by stimulation index) were observed in PBL, which were then followed by MLNL. Proliferative responses of lymphocytes isolated from local tissues were evaluated as an indicator of the local immunity both at the primary site of infection (LPL), and in the MLN which drain this site and are themselves a secondary site of infection. LPL and MLNL were compared with circulating PBL. The responses by PBL, MLNL, and LPL were consistent on the basis of presence/absence, if not in magnitude. It would appear that the local immunity is reflected in the PBL responses: animals with positive MLNL and LPL responses also had positive PBL results. However the presence of a positive PBL response did not equate to a positive response by MLNL or LPL.

One animal of the tuberculoid group (P34), and three animals from the lepromatous group had positive PBL responses, but a negative MLNL reponse. Likewise of the eleven control animals which had positive proliferation by PBL, only two were negative for MLNL. On the other hand, only one of all animals examined had a positive MLNL result with a negative PBL result, and this was sheep P30 of the lepromatous group which had a negative PBL SI of 1.02, and a low positive MLNL SI of 3.6.

All animals which had a positive LPL proliferation assay result also had positive results for both MLNL and PBL. Two of the tuberculoid cases examined for LPL responses were negative, but both had a positive PBL response. Of the lepromatous

group, only one animal had any positive proliferation by LPL and this was sheep P40, which similarly had high PBL and MLNL responses. LPL responses, however, were otherwise of very low magnitude, indicating poor lymphocyte proliferation by cells of lamina propria origin. The magnitude of the responses may have been influenced by differences in the cell populations assayed. Each tissue sample was likely to be constituted by different proportions of cell types. In chapter 4, it was noted that tuberculoid lesions of the ileum were associated with higher percentages of $CD4^+$ cells, and that the PBL of infected animals contained higher percentages of B cells, with MLNL differing only in the percentages of $\gamma\delta$ TCR^+ T cells, which were found to be higher in lepromatous cases. Differences in APC type, frequency and capacity may exist between the groups of animals, and between the tissues, and therefore may have resulted in differing levels of proliferative responses. Circulating PBL may be expected to be of a wider antigenic repertoire with resultant increased scope for antigenic cross-reaction, and this may explain higher proliferative responses from these cells. Therefore, the magnitude of response may not necessarily be purely a reflection of the relative numbers of memory cells in these populations, but may be a function of a number of variables. The populations are not uniform, and so neither can a direct comparison be made, nor a response of similar magnitude be expected.

A more standardised and more readily quantifiable protocol would have involved the preparation of autologous APCs for each animal. However, this would have been possible only in a truly experimental-infection situation, as the opportunity for preparation of autologous APCs did not present when using naturally infected animals which were in the terminal stages of disease. Nonetheless, the purification of the cells prior to assay was consistent, with all isolated cells being subjected to lymphoprep centrifugation. Selective depletion of cell types was therefore likely to be the same for each tissue. These results indicate that proliferative responses of PBL in LSA tests provide an indication of the immune status at the site of infection, and are reflected in

the positive correlations between PBL and MLNL responses in the control group, and the MLNL and LPL responses of the tuberculoid group.

LPL have been found not to proliferate strongly in response to antigen stimulation, but yet are considered to be highly specific memory cells providing helper function, and detection of cytokine elaboration of has been proposed as a more effective measurement of antigen-specific response from these cells (Zeitz *et al.*, 1991). In keeping with this observation, LPL had the lowest of all proliferative responses. However, the APC numbers and capacity were not determined, although on LPL isolation high numbers of cells were present which corresponded on FACSscan scatter profiles to cells of the monocyte/macrophage lineage.

Comparison on the basis of pathological group (which was determined as described in chapter 2 on the basis of lesion type and bacillary load, and in chapter 3 was related to the lymphocyte population) showed that cells isolated from all three tissues of the tuberculoid group had significantly higher responses than those from control animals. This was the case for PBL, MLNL, and LPL, and suggests an increased antigen-specific proliferative capacity in all three tissues. This could be related to the alterations in the relative proportions of the lymphocyte subsets in this group of animals. Interestingly, median antigen-specific proliferative responses of the lepromatous group were found to be lower than those of the control group (although not statistically significant), and this may be considered to be an indication of depressed immune responses, be it of mycobacterial or host induction. The contrast in the proliferative responses of the lepromatous and tuberculoid groups is striking in that the lepromatous group responded very poorly to antigen. The relative proportion of subsets may have an effect on the magnitude of the proliferative response, however altered functional and proliferative capacity would be more likely to account for this than absolute numbers of T-cell subsets. Host and mycobacterially derived suppressor

factors have been described. Chemical constituents of the cell walls from some mycobacterial species inhibit intracellular killing by macrophages (Kaufmann, 1993; Britton *et al.*, 1994). This phenomenon has been described for *M. tuberculosis*, *M. leprae* and *M. avium.* Hines *et al.*, (1993) isolated three macrophage inhibitory factors (MIF) from *Map* that inhibited intracellular killing by bovine macrophages, and characterised these as glycolipid compounds. In addition to a direct effect on macrophages, a depressive effect by mycobacterial antigens on lymphocyte proliferative responses has been described in humans (Fournie *et al.*, 1989) and similarly, in the mouse after administration of *M. avium* antigens (Brownback and Barrow, 1988). Depressed intracellular killing of bacilli in *Map* infected bovine monocytes has been reported, resulting in failure to achieve elimination of phagocytosed bacteria, with the addition of recombinant IFNγ having no effect on intracellular bacillary growth (Zurbrick *et al.*, 1988). *M. leprae* infected macrophages have been shown to be be refractive to upregulation by IFNγ (Sibley and Krahenbuhl, 1987) and this phenomenon appears to be attributable to LAM (Sibley *et al.*, 1988). By extension, macrophages in multibacillary, lepromatous type infections, may similarly have functionally impaired or depressed APC capability as a consequence of the high numbers of mycobacteria, and thus result in reduced lymphoproliferative responses.

Lymphocyte proliferative responses have been examined and found to be lowered in the case of lepromatous leprosy (Modlin *et al.*, 1988). Kreeger and Snider (1992), and Kreeger *et al.*, (1992) described lowered PBL lymphoblast proliferative responses in paratuberculosis of cattle. Likewise Chiodini and Davis (1992) described a period of lowered *in vitro* proliferative responses following vaccination with *Map* antigen, which were subsequently re-established 5-6 months post immunisation. A role was described in this work for the involvement of γδ TCR^+ cells in the response to *Map* antigens, and by other workers in the initial response to mycobacteria (Griffin

et al., 1991; Ladel *et al.,* 1995). A putative role for the γδ TCR^+ T-cell subset has been proposed in the early stages of mycobacterial infection as a bridging response before the αβ T-cell subsets have begun clonal expansion. Pollock *et al.*, (1996) performed a sequential analysis of circulating PBL sub-populations in *M. bovis* infection, and described a decrease in the number of circulating γδ TCR^+ T cells, with eventual dominance of the $CD8^+$ subset. Zhao and Collins, (1994), described a similar phenomenon in the response to experimental infection of calves with *Map*, and suggested that this coincided with a recruitment of γδ TCR^+ cells to the site of infection. Chiodini and Davis, (1992), described a suppressive role for γδ TCR^+ cells on proliferative responses by means of a cytotoxic effect on $CD4^+$ cells. The concept of suppression by T cells is recognised within the context of mycobacterial infections, with suppressor T cells having been isolated and cloned, in particular from leprosy patients (Modlin *et al.,* 1984; Ottenhoff *et al.,* 1986). Various mechanisms whereby suppression is achieved have been described, however in the light of the functional delineation of $CD4^+$, and more recently of $CD8^+$ T cells into groups according to their patterns of cytokine production (Salgame *et al.,* 1991), the traditional concepts of T cell suppression have largely been revised (reviewed by Bloom *et al.,* 1992). Reduced proliferative responses in lepromatous mycobacterial disease may be due to the presence of high numbers of mycobacteria which are themselves suppressing cellular proliferation. Alternatively, suppression may be a result of the host's attempt to achieve a damping-down effect on high immunity, DTH responses seen in the tuberculoid form of the disease, to an antibody, or type 2 response as a means of limiting tissue damage. This would to some extent explain the diminished proliferative response and the coincident antibody response, which is largely ineffective in achieving protection and clearance of the pathogen. It is likely that each of these suppressive mechanisms occurs to some extent, and that each is important in the balance between host and pathogen.

The role of APCs in the immunopathogenesis of paratuberculosis ought not to be overlooked. Both APC numbers and APC type are likely to be of fundamental importance in the elicitation of an immune response. Likewise, secondary signals are required (in addition to the primary antigen-specific signal via the TCR/CD3 complex) for effective T-cell activation and may take the form of cytokine signal or of cell-surface costimulatory molecules. Inadequate costimulatory signals have been shown in other systems to lead to apoptosis, or clonal anergy, with subsequent refractivity to antigenic stimulus (reviewed by Schwartz, 1990). Professional APCs of myeloid origin are capable of providing co-stimulation via interaction of B7-1 and B7-2 cell-surface molecules with their ligand (CD28) on T lymphocytes. Likewise, elaboration of cytokines may be sufficent to provide a costimulatory signal. Non-professional (of non-myeloid lineage) APCs may influence the outcome of the immune response (reviewed by Nickoloff and Turka, 1994), perhaps predisposing the nature of the immune response to that of either Th1 or Th2 type, and Th2 type responses may be misinterpreted as anergy if Th1 effector mechanisms alone are assayed. A role has been described for M cells in the pathogenesis of paratuberculosis as a result of uptake of *Map* into the mucosa (Momotani *et al.*, 1988). Chiodini (1991) discussed antigen presentation in the mucosal environment in paratuberculosis. M cells are thought to transport antigen to basally-apposed macrophages which can then in turn present antigen to $CD4^+$ cells. In contrast, enterocytes have been considered to be non-professional APCs with the ability to present antigen in conjuction with MHCI directly to $CD8^+$ cells, and selective activation of $CD8^+$ cells has been hypothesised via this mechanism (Chiodini, 1991). In addition, antigen presentation is possible by B cells, and B cells may preferentially direct $CD4^+$ cells to the Th2 pathway (Finkelman *et al.*, 1990). $CD5^+$ B cells may act as APCs for helper T cells (Carson *et al.*, 1991). Cells of this subset occupy the mantle zone in lymphoid follicles, thus permitting contact with T cells, and $CD5^+$ B cells may be instrumental in activation of type 2, suppressor T cells (Bloom *et al.*, 1992). Peyer's patches are considered to be the portals of entry

of infection, and are described as having a Th2 type environment which results in a strong drive towards antibody production at the mucosal surface and within the gut microenvironment (Daynes *et al.,* 1990).

CMI is required for clearance of the pathogen, and failure to achieve clearance suggests ineffective CMI effector mechanisms. CMI correlates with protection in leprosy (Yamamura *et al.*, 1991). In general, an inverse relationship exists between CMI and mycobacterial burden. Conversely, humoral immunity has been considered to be related to high mycobacterial numbers. However, significant immune-mediated tissue damage was found to exist in the tuberculoid cases examined in this study, and may be a result of strong DTH response in these animals. A balance must exist between high immunity and DTH of a magnitude which will result in tissue damage. It has been suggested that an initial Th1 response is necessary for DTH and protection in mycobacterial infections, but a subsequent Th2 type response is switched on as a means of limiting inflammation and thereby minimising tissue injury, particularly in tuberculosis (Fenton and Vermeulen, 1996). However, Th2 responses may favour immunosuppression in cases of advanced disease. It is interesting that no wider dissemination and multiplication of *Map* seems to occur in non-gut tissues of lepromatous cases given that these animals appear to have poor antigen-specific immune responses, even by PBL. This may be due in part to the nutrient requirements of *Map*, or perhaps to decreased migratory potential by host macrophages.

It is clear that, as in other mycobacterial diseases (Modlin, 1994), a spectrum of pathological manifestations exists which appears to be inextricably linked with the host immune responses to the pathogen. The tuberculoid lesions correspond with resistance to the pathogen with containment of bacillary growth and limited lesions, but with tissue damage in evidence. However, the lepromatous lesions are coincident with immunological susceptibility to the pathogen signalled by extensive bacillary growth

and multiple lesions. This is demonstrated by the relation of histological lesions in paratuberculosis to the degree of *in-vitro* cellular proliferation to *Map* antigen.

CHAPTER SIX

EFFECT OF T-CELL SUBSET DEPLETION ON THE LYMPHOCYTE PROLIFERATIVE RESPONSE

6.1 INTRODUCTION

Roles have been described for the $CD4^+$, $CD8^+$, and $\gamma\delta$ TCR^+ T-cell subsets in the protective response to mycobacterial infection. It was considered that the *in-vitro* lymphoproliferative responses of individual T-cell subsets to JPPD would merit further examination, and that this may provide information on which subset is primarily involved in proliferation in response to JPPD. It may be expected that MHCII-restricted $CD4^+$ T cells would be the subset most likely to proliferate in response to soluble, proteinaceous antigen which is likely to be processed and presented by APC on MHCII via the endoplasmic route. However, proliferation of $\gamma\delta$ TCR^+ T cells in response to soluble mycobacterial antigens has also recently been described (Esin *et al.,* 1996). Furthermore, suppressive roles have been suggested for $\gamma\delta$ TCR^+ and $CD8^+$ lymphocytes in paratuberculosis (Chiodini and Davis, 1992; Chiodini and Davis, 1993), and removal of either of these subsets may result in increased proliferation by the remaining cells. In order to investigate differential proliferative responses of T-cell subsets in response to *Map* antigen, Magnetic Activated Cell Sorting (MACS) techniques were employed to deplete the PBL population of individual subsets. MACS technology permits simple and relatively rapid separation of T-cell subsets, and the resultant cell populations have been shown to be compatible with inclusion in functional assays (Abts *et al.,* 1989; Jacobs *et al.,* 1993). MACS is based on the principle of attaching biodegradable, magnetically-charged microbeads, of 50-150 nm in diameter, onto target cells. This can be achieved by indirect staining of target lymphocytes by labelling with a primary MAb specific for

the desired cell-surface marker, followed by an isotype-specific, microbead-conjugated, secondary antibody. Labelled cells are retained within the ferromagnetic, stainless steel matrix when in the magnetic field of the MACS apparatus (Miltenyi *et al.,* 1990). MACS was performed on cells isolated from peripheral blood from paratuberculosis cases. The isolated cell suspensions were subjected to negative selection by depletion of single T-cell subsets and subsequently assayed in LSA. In cases of negative selection of PBL, only the labelled cells are removed, and the residual cells remain, and still contain APCs. In negative selection, the potential for impairment of proliferative responses is minimised since the MAb and microbead labelled cells are not included in the assay, and there is no need for preparation of autologous APCs. MACS facilitates rapid depletion of cellular subsets on phenotypic basis, with minimal functional impairment and very high levels of specificity and purity.

6.2 MATERIALS AND METHODS

6.2.1 Animals

Five sheep with clinical paratuberculosis, and in addition nine goats from a herd with endemic paratuberculosis (chapter 9) were chosen for this study. The goats had either histological evidence of paratuberculosis infection, or serological evidence based on the presence of *Map*-specific serum antibody, or were previously determined on at least one occasion to have positive PBL proliferation in response to Johnin PPD.

6.2.2 Isolation of lymphocytes

PBL were isolated as for FACS analysis (described in section 4.2.5), and an aliquot of cells reserved for MACS. Isolation of afferent lymph cells by cannulation of MLNL afferent lymph ducts was attempted without success, and efferent lymph which was successfully collected on cannulation of efferent ducts did not contain sufficient numbers of APCs to permit proliferation in LSAs.

6.2.3 MACS protocol

MiniMACS apparatus (Miltenyi Biotech, Camberley, Surrey) was used for this procedure. MiniMACS columns have a capacity for binding approximately 1 x 10^7 positively labelled lymphocytes. The MiniMACS column was placed in the separation apparatus and a flow-rate limiter (26 gauge needle) attached. All procedures were performed in a Class II laminar flow hood to preserve sterility. Immediately before use, the column was washed by filling with 500 µl MACS buffer (PBS containing 5mM EDTA, and 0.5% FCS to prevent non-specific binding to the column) and allowing it to flow through.

Cell suspensions were washed by centrifugation in MACS buffer prior to labelling. 1.5-2.0 x 10^7 cells were resuspended and incubated with monoclonal antibodies for 30 minutes at 4°C. MAb used were SBUT4, SBUT8, and 86D (table 4.1) for labelling of ovine and caprine (Davis and Ellis, 1991) $CD4^+$, $CD8^+$ and $\gamma\delta$ TCR^+ lymphocyte subsets respectively. Quantities and dilutions of MAb were calculated from those titrated for FACS analysis, and were verified by staining parallel cell samples for cytofluorimetry which were not subjected to MACS. Cells were then washed by centrifugation in 10 ml MACS buffer to remove excess MAb. Cells were

resuspended in 80 µl MACS buffer and 20 µl anti-mouse-IgG conjugated MACS microbeads (Miltenyi Biotech) were added, and the suspension incubated at 4°C for 15 minutes before the wash step was repeated. In order to verify depletion, cells were resuspended in 300 µl of 1:100 dilution FITC-conjugated rabbit anti-mouse $F(ab')_2$ (Dako), and the cell suspension incubated for 10 minutes at 4°C in the dark. Washing was repeated to remove excess FITC-conjugated antibody, before the cells were resuspended in 500 µl MACS buffer.

The cell suspension was subsequently pipetted onto the MiniMACS column and allowed to run through the column completely, using the 26 gauge needle as flow-rate limiter. The MiniMACS column incorporates a 'flow stop' design in order to prevent the premature elution of bound cells. In order to remove any non-labelled, suspended cells, the column was washed by pipetting 500 µl of MACS buffer onto it and allowing it to run through. The effluent was collected as unbound cells into a 15 ml centrifuge tube. The flow resistor was the removed and the column washed twice using two 500 µl volumes of MACS buffer. Bound cells were eluted by removing the column from the magnetic field and adding 500 µl of MACS buffer to the column and applying positive pressure using the plunger supplied. All cells were washed by resuspension in RPMI and centrifugation. This step was repeated, and both bound and unbound cell suspensions were counted. Samples were taken from unsorted, bound and unbound cell populations for FACS analysis in order to verify that cell sorting had been successful. For this, 1×10^5 cells were aliquoted into FACS tubes and were prepared and analysed by flow cytometry as described in section 4.2.

6.2.4 LSA

MACS-bead stained cells which were not subjected to sorting, and T-cell subset depleted samples were subjected to LSA. LSAs were performed according to the protocol described in section 5.2.4, and the results expressed as stimulation indices for each group of cells (unsorted and depleted populations).

6.2.5 Statistics

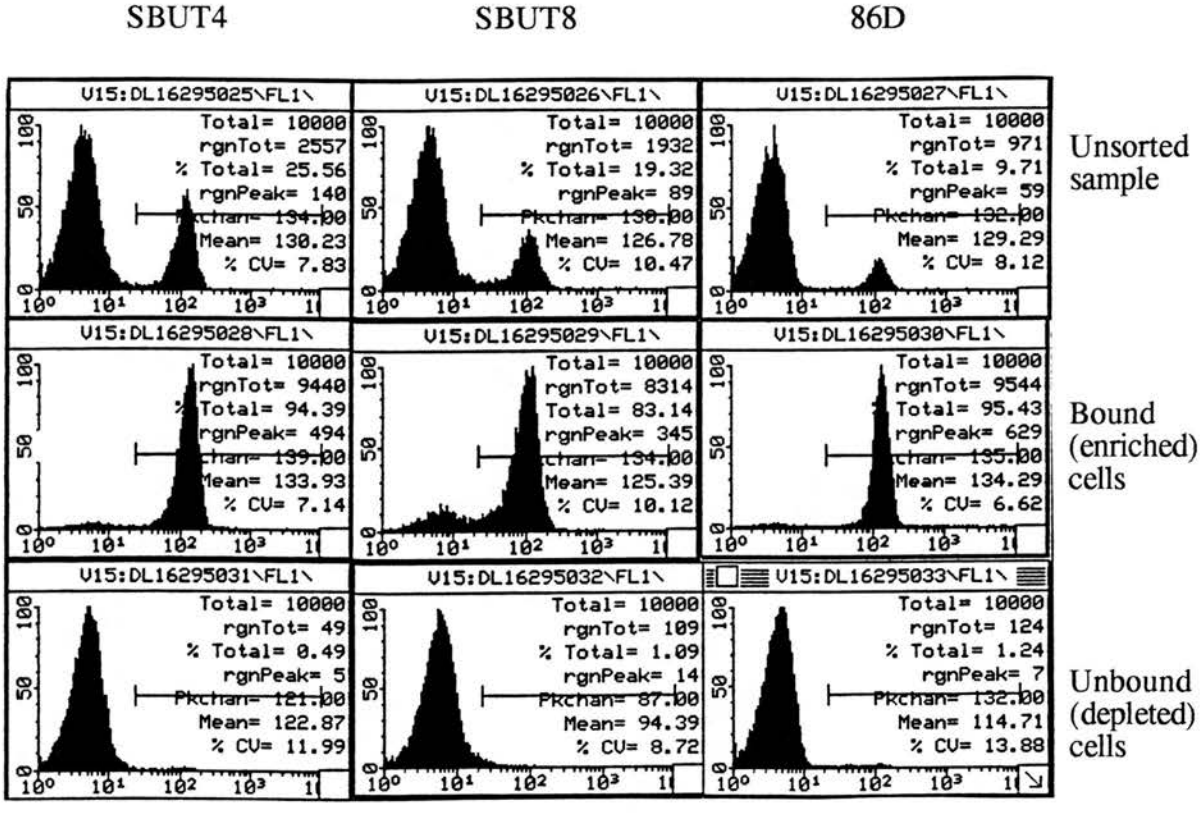
Differences of the paired data were calculated by subtracting the SI of the depleted population from that of the unsorted population. Data did not conform to the normal distribution, and so the Wilcoxon test was applied to the differences and used for the determination of the 95% confidence intervals (CI) of the SI values. Significance was considered to be $P<0.05$.

6.3 RESULTS

6.3.1 Depletion of T-cell subsets by MACS

High levels of purity were verified for both depleted and bound cells for all three MAbs when cell samples were analysed by flow cytometry (represented in figure 6.1).

Figure 6.1: Representative FACScan histograms of caprine PBL (goat A062) which have been subjected to MACS staining and sorting. Three distinctive cellular populations are shown for each of the three MAbs used (SBUT4, SBUT8 and 86D): unsorted cells stained with MACS microbeads but not applied to the MACS column; cells bound within the column; and the final population which has been depleted of positively stained cells. Good levels of T-cell-subset depletion are apparent after a single passage through the column.



6.3.2 LSA results for depletion of $CD4^+$ cells.

The median SI values of pre-MACS and depleted populations and their 95% confidence intervals are shown in table 6.1. SI values for individual animals are included in appendix 6.1.

A significant reduction in the SI of the $CD4^+$ depleted population (median SI value 1.68) was found compared with the unsorted population (median SI value 3.33) (median difference in SI 2.28, $P<0.05$). It would therefore appear that removal of the $CD4^+$ subset is associated with a decrease in cellular proliferation, as measured by incorporation of tritiated thymidine, and suggests that this subset is instrumental in the proliferative response to JPPD. However, the magnitude of proliferative responses was low, especially within the group of goats.

6.3.3 LSA results for depletion of $CD8^+$ and $\gamma\delta$ TCR^+ cells.

Examination of both the $CD8^+$ and $\gamma\delta$ TCR^+ paired samples revealed more equivocal results, with some animals showing increased, and others decreased proliferative responses from depleted samples. Analysis by the Wilcoxon test, however, showed that the net effect of this resulted in no significant differences in the paired data. No apparent difference was noted in the median SI of the $CD8^+$ depleted samples (2.93) compared with that of the unsorted populations (2.75), nor between the median SI values of $\gamma\delta$ TCR^+ depleted and unsorted populations (median values 2.20 and 2.99 respectively). If no significant difference exists between the pairs of SI values, then the median of the differences will tend towards zero, with the samples from any population being distributed around the median, and this appeared to be the case for the $CD8^+$ and $\gamma\delta$ TCR^+ paired samples.

Table 6.1: Median and 95% CI values for the SI of unsorted and depleted populations.

MAb	n	Unsorted	Depleted	Difference
SBUT4	10	3.33 (1.8,31.1)	1.68 (1.1,3.6)*	2.28 (0.1,27.4)
SBUT8	9	2.93 (1.91,7.05)	2.75 (1.7,12.9)	-0.14 (-8.8,4.0)
86D	12	2.20 (1.53,5.14)	2.99 (2.22,9.79)	-0.41 (-4.42,0.53)

Significant difference in the median SI values is denoted * ($P<0.05$).

6.4 DISCUSSION

MACS techniques appear to be useful for the depletion of T-cell subsets from ovine PBL, with high levels of purity observed in both the depleted and enriched populations. It would appear to be preferable in terms of efficiency, speed and cost to other established methods of cell sorting such as panning and FACS sorting.

The results of this study confirm that the $CD4^+$ subset is the one which is most likely to respond by proliferation to JPPD antigen. Because of the peptide nature of PPD, it is likely that antigens are presented in association with MHCII, having been processed via the endosomal pathway, and hence the cells which are likely to 'see' and respond to presented antigen are MHCII-restricted $CD4^+$ cells (reviewed by Janeway, 1992).

When preparing the LSA, cells were aliquoted in constant numbers into the assay (2×10^5 per test well), and therefore depletion of the $CD8^+$ and $\gamma\delta$ TCR^+ subsets must by definition result in enrichment of $CD4^+$ cells, and this may be the cause of the increase in SI observed in several of the $CD8^+$ and $\gamma\delta$ TCR^+ depleted samples. Paradoxically, however, several of the $CD8^+$ and $\gamma\delta$ TCR^+ depleted samples had lowered SI. An improvement in the experimental protocol would be to avoid the selective enrichment of the non-depleted subsets by adjusting the number of cells placed into the LSA. Subtraction of the percentage of cells which had been depleted would achieve this (for example, if $CD8^+$ cells were depleted and this subset equated to 20% of the population, then in total 80% of 2×10^5 negatively selected cells would be placed into the LSA instead).

The work of Chiodini and Davis (1992 and 1993) suggested that $CD8^+$ and $\gamma\delta$ TCR^+ subsets have an immunoregulatory role in the proliferative response to *Map* antigen, with $\gamma\delta$ TCR^+ T cells exerting a cytotoxic effect on $CD4^+$ cells, thereby

reducing the response of this subset. The γδ TCR$^+$ lymphocytes were themselves also considered to be subject to regulation by CD8$^+$ cells through an undefined mechanism. The experiments described were based on one experimentally immunised bovine animal, and were correlated with data from the PBL of clinically affected cattle. In this group of sheep and goats, no pattern was evident on depletion of either of the CD8$^+$ and γδ TCR$^+$ subsets. However, a wide range of pathological and immunological manifestations was present in the group of animals in this study, and as such clearly defined responses may not necessarily be expected. More consistent results may have been observed if the animals examined had conformed to a single group, for example, animals with tuberculoid or with lepromatous lesions, or with strong CMI responses.

Further delineation of the proliferative responses of each subset may be possible by performing experiments in which autologous, irradiated APC were prepared, and reconstitution of the proliferative response examined by adding positively selected T-cell subsets. In addition, γδ TCR$^+$ and CD8$^+$ lymphocytes have been shown to respond to live mycobacteria. Boom *et al.* (1990), and Havlir *et al.* (1991) described the specific expansion of γδ TCR$^+$ T cells by *M. tuberculosis*-infected macrophages. However, Esin *et al.* (1996) described proliferation of γδ TCR$^+$ cells in response to live, killed and soluble extracts of *M. tuberculosis* and *M. avium*, which to some extent is in contradiction with the findings of Boom *et al.* (1990), who noted expansion of this subset solely in response to live mycobacteria. This disparity may be a function of mycobacterial strain differences used in the respective assays. Expansion of the CD8$^+$ subset has been found to be predominantly in response to live mycobacteria (Esin *et al.*, 1996; Kale Ab *et al.*, 1990). Experimental infection of monocytes with live *Map* and use of these cells as APCs in proliferation assays may provide further information on the responses seen *in vivo* in infected animals.

CHAPTER SEVEN

INTERFERON-γ AND INTERLEUKIN-2 PRODUCTION IN PARATUBERCULOSIS

7.1 INTRODUCTION

Intracellular bacterial infections are characterised by the exploitation of the intracellular environment for survival and growth (reviewed by Kaufmann, 1993). Intracellular bacteria are in a position of privilege, permitting evasion of humoral immune factors, and of the direct intervention of scavenging PMN granulocytes. In such a situation, T lymphocytes are crucial in the immune response to intracellular bacteria, both in the surveillance of antigen presented by the host cell, and in T cell effector mechanisms, the principal of which is the activation of the macrophage. This converts the permissive habitat for the pathogen into one which is ultimately capable of killing it. This is achieved by T-cell cytokine elaboration and activity, both in the upregulation of the macrophage cytotoxic capacity, and also in the promotion of granuloma formation. Granulomas serve both for containment of infection, and provide the microenvironment for aggressive DTH and antibacterial activity. Unfortunately, in many intracellular bacterial infections, aseptic clearance is not always achieved and chronic disease ensues, which is frequently exacerbated by the host's cytokine activity.

Protection is mediated by cell-mediated immunity and more specifically the DTH response. The DTH response to intracellular pathogens would be expected to involve the elaboration of IFNγ and IL-2 in particular by T cells, as these are considered to be the primary cytokines responsible for the promotion of CMI. In the murine model, Th1 cells and specifically their production of IFNγ, have been shown to be

fundamental to the DTH response (Cher and Mosmann, 1987; Fong and Mosmann, 1989). In the mouse, in which Th1 cells have been most clearly characterised, this helper-cell subset have been shown to produce IFNγ, IL-2 and TNF-β, but not IL-4, IL-5, IL-6 and IL-10, which are considered to be Th2 cytokines (reviewed by Mosmann and Coffman, 1989). This concept has in recent years been expanded to include similar patterns of cytokine production by $CD8^+$ and γδ TCR^+ cells (designated type 1 and type 2 cells respectively) (reviewed by Mosmann and Sad, 1996), and also to humans in various infectious and non-infectious diseases (reviewed by Romagnani, 1994). Similar patterns of Th function have been suggested for other mammalian species including ruminants (Brown *et al.,* 1993).

IFNγ is a cytokine produced primarily by T cells in response to antigenic stimulation (Havell *et al.,* 1982), and has several biological properties relevant to the clearance of intracellular infections. It is instrumental in the upregulation in macrophages of reactive oxygen and nitrogen intermediates (Nathan *et al.,* 1983), thereby resulting in the restriction of growth of mycobacteria or increasing intracellular killing (Rook *et al.,* 1986; Nathan *et al.,* 1986). In addition, IFNγ results in the upregulation both of MHCII expression by macrophages and other cell types, and of ICAM-1 (Dustin *et al.,* 1988), resulting in increased potential for interaction between T cells and accessory cells. IFNγ also influences the differentiation of T cells into Th1 (or type 1) cells (reviewed by Mosmann and Sad, 1996). IL-2 promotes T-cell proliferation and thereby clonal expansion, and in turn may stimulate the production of IFNγ (Kasahara *et al.,* 1983) eventually resulting in the elimination of the pathogen. IL-2 essentially governs lymphoproliferative responses of Th1 cells. On the evidence of increased proliferation by lymphocytes of tuberculoid cases it would be expected that these cytokines might be dominant in that situation. Assaying the levels of IFNγ and IL-2 produced by T cells in response to mycobacterial antigen in effect gives an indication of the protective response. Tuberculoid cases would therefore by definition

be expected to be highly DTH in character, corresponding to protective immunity. Lepromatous cases, however, which showed poor lymphoproliferative responses, and allowed persistence and growth of intracellular bacteria, may be expected to have lower levels of these protective, DTH cytokines. A positive correlation would be expected between T cell proliferation and IFNγ and IL-2 activity. Indeed the lepromatous group may be expected to have low levels of IFNγ and IL-2 due to the hypothesis of suppression and/or type 2 responses. Local injection of IFNγ and IL-2 has in addition been shown to re-establish cell-mediated immunity in lepromatous lesions of leprosy, resulting in lowered mycobacterial burdens (Nathan *et al.,* 1986; Kaplan *et al.,* 1989).

In contrast, down-regulation of Th1 responses may be a result of Th2 cell activity, mediated by the cytokines IL-4 and IL-10 (Mosmann and Sad, 1996). IL-4 promotes B-cell differentiation and immunoglobulin class switching (Yokota *et al.,* 1986) which may result in higher levels of serum antibody in lepromatous cases, and is responsible for the differentiation of Th2 lymphocytes (Fernadez Botran *et al.,* 1988; reviewed by Mosmann and Sad, 1996). IL-4 and IL-10 in addition have a negative immunoregulatory effect on CMI, and on Th1 cell-subsets in particular. IL-4 blocks IL-2-induced proliferation by down-regulation of IL-2 receptors (IL-2r) (Martinez *et al.,* 1990), and blocks the IFNγ-induced activation of macrophage intracellular killing (Lehn *et al.,* 1989, Liew and Cox, 1991). IL-4 has also been suggested as a significant mediator of T-cell suppression (Bloom *et al.,* 1992). IL-10 has been shown to downregulate both IFNγ production and cell-surface costimulatory molecules in human tuberculosis (Gong *et al.,* 1996), and when administered to *Listeria monocytogenes*-infected mice was seen to result in immunosuppression and the ablation of innate resistance to the pathogen (Kelly and Bancroft, 1996). Immunosuppressive effects of IL-10 have also been noted in leishmaniasis (reviewed by Kemp *et al.,* 1996).

In order to further delineate the functional responses of lymphocytes isolated from animals affected by paratuberculosis, it was decided to assay the cytokine production of cells isolated from tissues and cultured with paratuberculosis antigen. The earliest measurable immunological responses to infection with intracellular bacteria in general are T-cell-mediated responses, which are characterised by T-cell proliferation and clonal expansion, and the elaboration of cytokine mediators (Kaufmann, 1993). IFNγ production has been previously found to correlate with T-cell proliferation (D'Andrea *et al.,* 1986). Assays for ovine IL-2 and IFNγ were available and were chosen as indicators of T-cell expansion and activation of CMI. The IFNγ assay is a MAb based sandwich ELISA, and was developed and used for the examination of IFNγ production in bovine tuberculosis (Rothel *et al.,* 1990), and has since been assayed for the diagnosis of bovine paratuberculosis (Billman-Jacobe *et al.,* 1992). Cross-reactivity with ovine IFNγ has been established (Rothel *et al.,* 1990; Entrican and Burrells, unpublished) and the test used for the detection of ovine IFNγ produced in response to *Map* and *Mas* antigen (Burrells *et al.,* 1995; Pérez *et al.,* 1994). The assay used to determine IL-2 activity in antigen-stimulated culture supernatants was an IL-2 bioassay, based on that described by Edfors-Lilja *et al.*, (1991), whereby IL-2 is required to induce proliferation in an IL-2-dependant cell line. For this purpose the bovine IL-2-dependant lymphoblastoid cell line BJ1004 (Reid *et al.*, 1989) was used.

7.2 MATERIALS AND METHODS

7.2.1 Sheep

Nineteen adult ewes clinically affected by paratuberculosis, and sixteen normal control sheep were used in this study. Animals were subjected to full necropsy examination (chapter 2) and on the basis of histopathological findings were classified as described into lepromatous (n=11) and tuberculoid (n=8) groups.

7.2.2 Collection of tissues and lymphocyte isolation

Blood, ileum and MLN were collected as previously described (section 4.2.2). PBL were isolated by Lymphoprep centrifugation (described in section 4.2.5). MLNL were isolated by cutting MLN into small pieces, and rubbing gently on small-gauge wire gauze immersed in RPMI-FCS media in a Petri dish, and the resultant cell suspension was collected by pipette. MLNL were then purified by Lymphoprep centrifugation (section 4.2.7) and were resuspended in RPMI-FCS. Ileal LPL were isolated and purified as described in detail in section 4.2.6. All cell suspensions were washed in RPMI-FCS by centrifugation at 650 x g, and resuspended in RPMI-FCS at a concentration of 1×10^6 ml^{-1} prior to aliquoting into assays.

7.2.3 Preparation of supernatants for testing

Supernatants of cell cultures stimulated by *Map* antigen were used to measure production of IFNγ and IL-2 under these conditions. These assays were performed by

Dr C. Burrells at the Moredun Institute. Whole blood stimulation was performed, and also cell suspensions resulting from isolation of LPL and MLNL were used. One ml of heparinised whole blood (10 iu heparin per ml of whole blood) was aliquoted into a well of a 24 well flat-bottomed multiwell plate, containing either 100 µl RPMI-FCS (negative control) or 100 µl Johnin PPD (section 5.2.4), and incubated at 37°C for 24 hours, after which plates were centrifuged at 450 x g to pellet the blood-cell fraction, and the culture supernatant collected. Supernatants were stored at -20°C and processed in batches in respective assays. The same procedure was adopted for the stimulation of MLNL and LPL using 2 x 10^6 cells suspended in 1 ml RPMI-FCS instead of the 1ml of whole blood. Resultant cell-culture supernatants were then subjected to the assays.

7.2.4 Interferon-gamma assay

A sandwich ELISA assay was performed (Rothel *et al.,* 1990) whereby 100 µl antigen culture-supernatant was added to wells in an ELISA plate (in triplicate) which had been pre-coated with anti-IFNγ MAb (IFN9) (Wood *et al.,* 1990). Plates were incubated at room temperature for 1 hour, washed, and then 100 µl of the second, horseradish peroxidase (HRP)-conjugated anti-IFNγ MAb (IFN2) was added and incubated for a further 30 minutes at room temperature. Plates were then again washed, and 100 µl of the colour-development substrates tetramethylbenzidine and hydrogen peroxide added. The colour development reaction was halted after 30 minutes by the addition of 50 µl of 0.5M sulphuric acid (H_2SO_4) to each well, and the optical density (OD) determined using a 'Titertek Multiskan' ELISA plate reader (Flow Laboratories, Irvine, UK) equipped with a 450 nm filter. Results were expressed as corrected OD (COD), and calculated by subtracting the OD of the well containing the medium control from that of the well containing the antigen. A positive cut-off level of COD = 0.05 was selected, and COD values above this level were considered positive.

7.2.5 Interleukin-2 assay

BJ1004 cells were washed three times in Iscove's Modified Dulbecco's Medium (IMDM), containing the same supplements as the RPMI for tissue culture (section 5.2.4). Cells were finally resuspended at a concentration of 1 x 10^5 ml $^{-1}$ and 100 µl of this cell suspension was aliquoted in triplicate into a 96 well flat-bottomed multiplate. To each well, 50 µl of cell-culture supernatant (or plasma) from the antigen stimulated cultures (section 7.2.3) was added, and then followed by 50 µl of IMDM to negative control wells. Plates were incubated (as described in section 5.2.4) for 72 hours, and at 16 hours before the end of the incubation were pulsed with 1 µCi of tritiated thymidine in 50 µl of IMDM. Sixteen hours later, cells were harvested and the uptake of tritiated thymidine measured using a beta counter (section 5.2.4). Stimulation indices were calculated by the same method as described, and a positive cut-off level of SI = 3.0 selected.

7.2.6 Statistics

Data were once again found not to conform to the normal distribution, and so non-parametric tests were used, in particular the Mann-Whitney confidence interval and test and the Spearman's Rank Correlation test. The IFNγ ELISA COD data-set contained zero values, and so were subjected to manipulation by addition of the constant 1 to remove zero values, before logarithmic (Log_{10}) transformation. Logarithmic transformation was applied to all data in order to facilitate graphical comparison of IFNγ and IL-2 values.

7.3 RESULTS

7.3.1 Observations on IFNγ and IL-2 production by individual animals

Results of the cytokine assays of individual animals are represented graphically in figures 7.1-7.6. A positive cut-off value of COD = 0.05 was assigned for the IFNγ ELISA and this corresponds to the logarithmic value of the manipulated data (Log_{10} (COD+1)) of 0.021. For the IL-2 bioassay, an LSA SI value of 3.0 was taken to be the positive cut-off, which in turn corresponds to a Log_{10} value of 0.477.

On examination of IFNγ and IL-2 production by PBL, animals of the infected groups which had positive levels of IL-2 production were also found to have positive levels of IFNγ (figure 7.4). One animal (P32) of the lepromatous group (6%), and 4 (50%) of the tuberculoid group (P23, P26, P27, XC39) had positive levels of SI in the IL-2 bioassay. In addition, however, a further three animals of those which had positive levels of IFNγ production were not associated with positive levels of IL-2 (P30 and P40 of the lepromatous, and P34 of the tuberculoid groups). In total three of the lepromatous group (19%) and five of the tuberculoid group (63%) were considered positive for IFNγ production (P30, P32, P40; and P23, P26, P27, P34, XC39 respectively). The same pattern however, was not observed for the control group, with only one animal (C44) noted to have a positive result for both IFNγ and IL-2 tests (figure 7.1). Of the control group, three animals (C36, C43, C44) gave positive IFNγ results (19%), and five animals (C44, C48, C49, C51, C52) gave positive IL-2 results (31%).

On examination of MLNL data, seven of the animals in the lepromatous group (64%) had significant IFNγ responses (P22, P30, P31, P32, P35, P37, P40), whereas only one was positive for IL-2 production (6%, P37) (figure 7.5). All

Figure 7.1: IFNγ and IL-2 elaboration by PBL isolated from individual sheep of the control group.

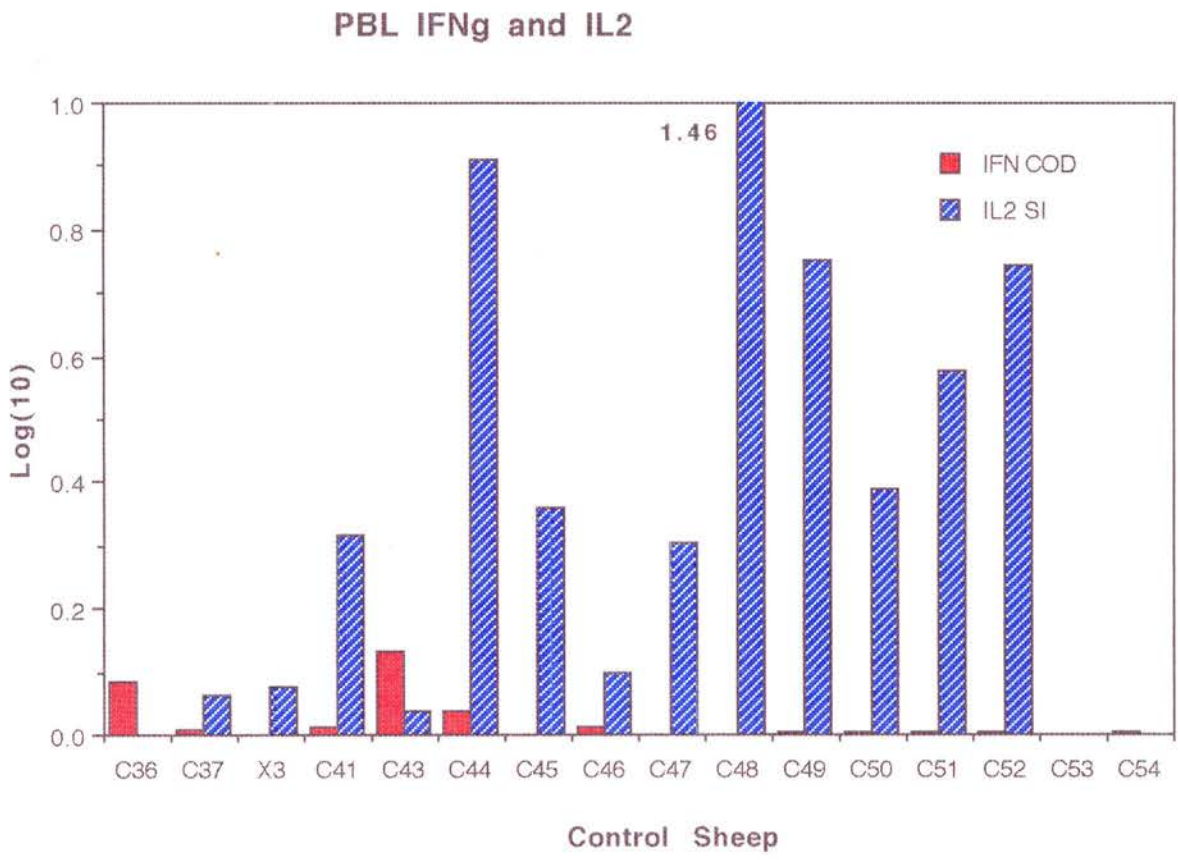


Figure 7.2: IFNγ and IL-2 elaboration by MLNL isolated from individual sheep of the control group.

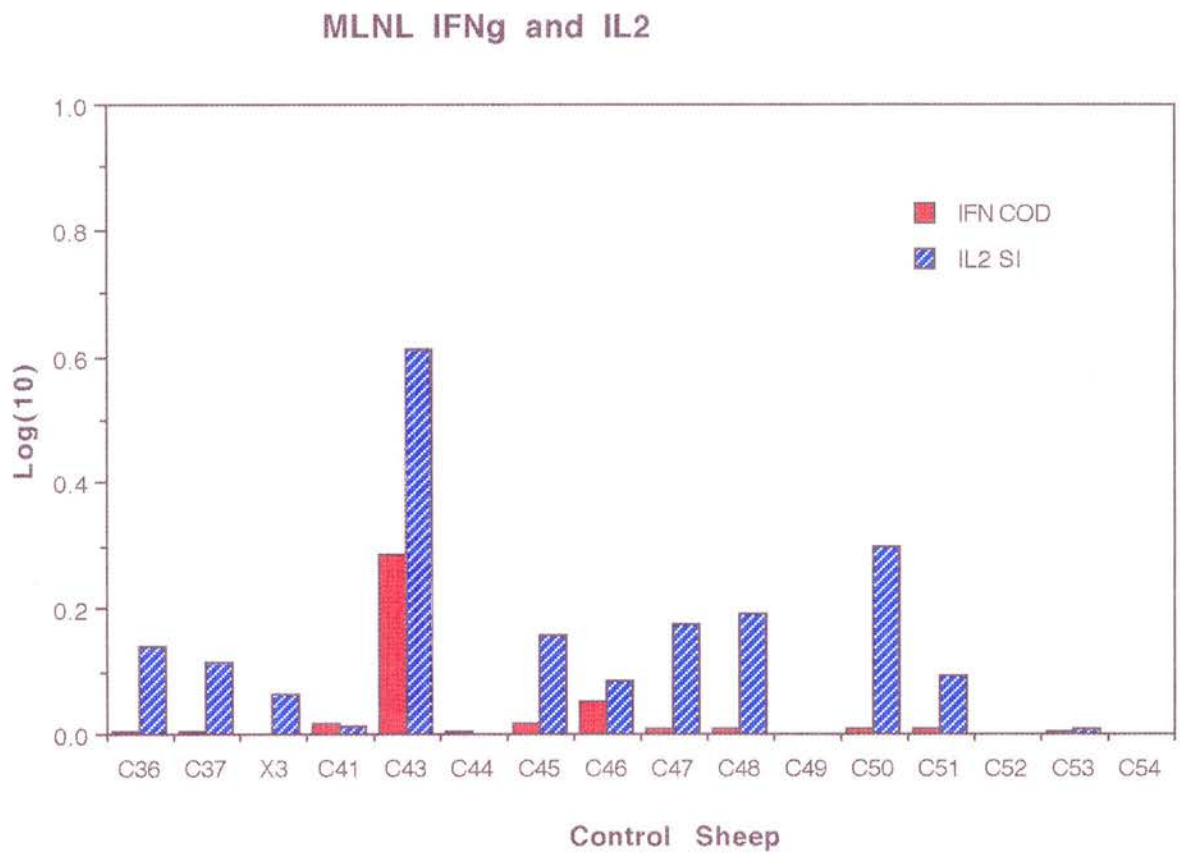


Figure 7.3: IFNγ and IL-2 elaboration by LPL isolated from individual sheep of the control group. ND: not determined.

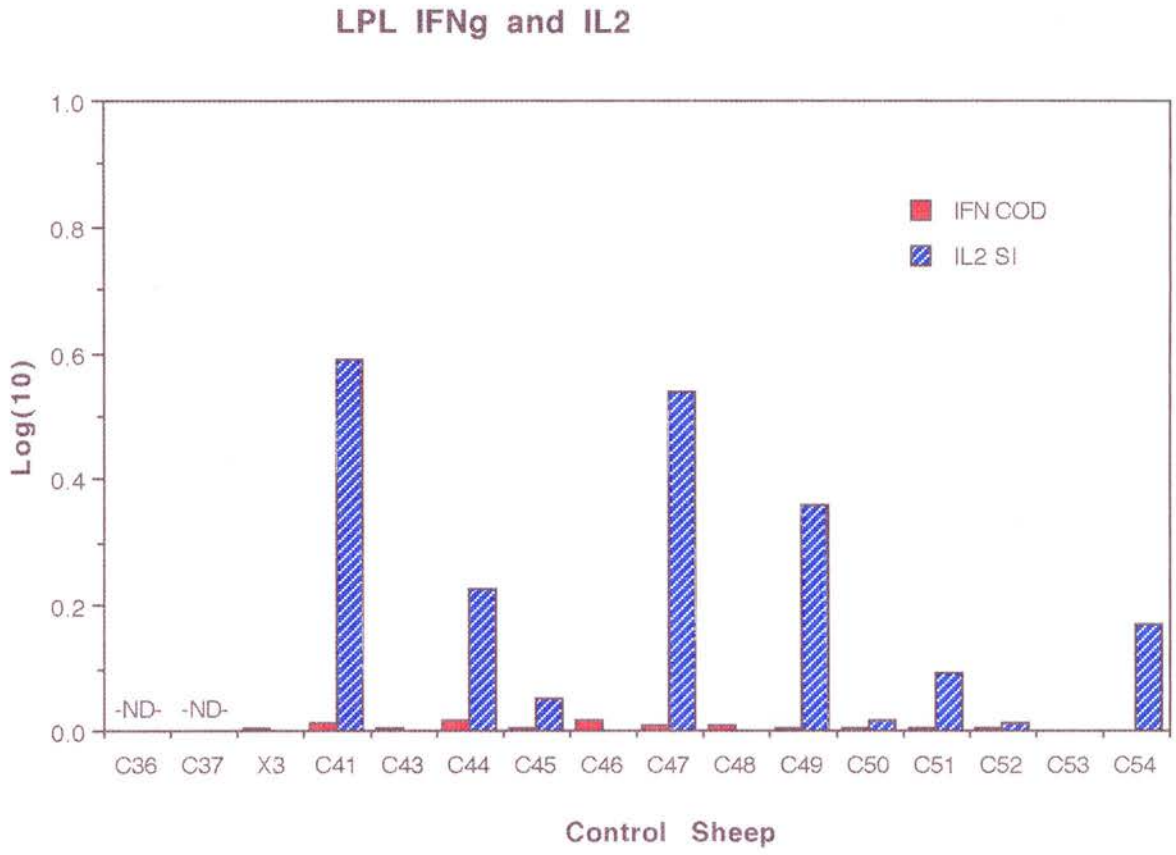


Figure 7.4: IFNγ and IL-2 elaboration by PBL isolated from individual infected sheep of the lepromatous and tuberculoid groups.

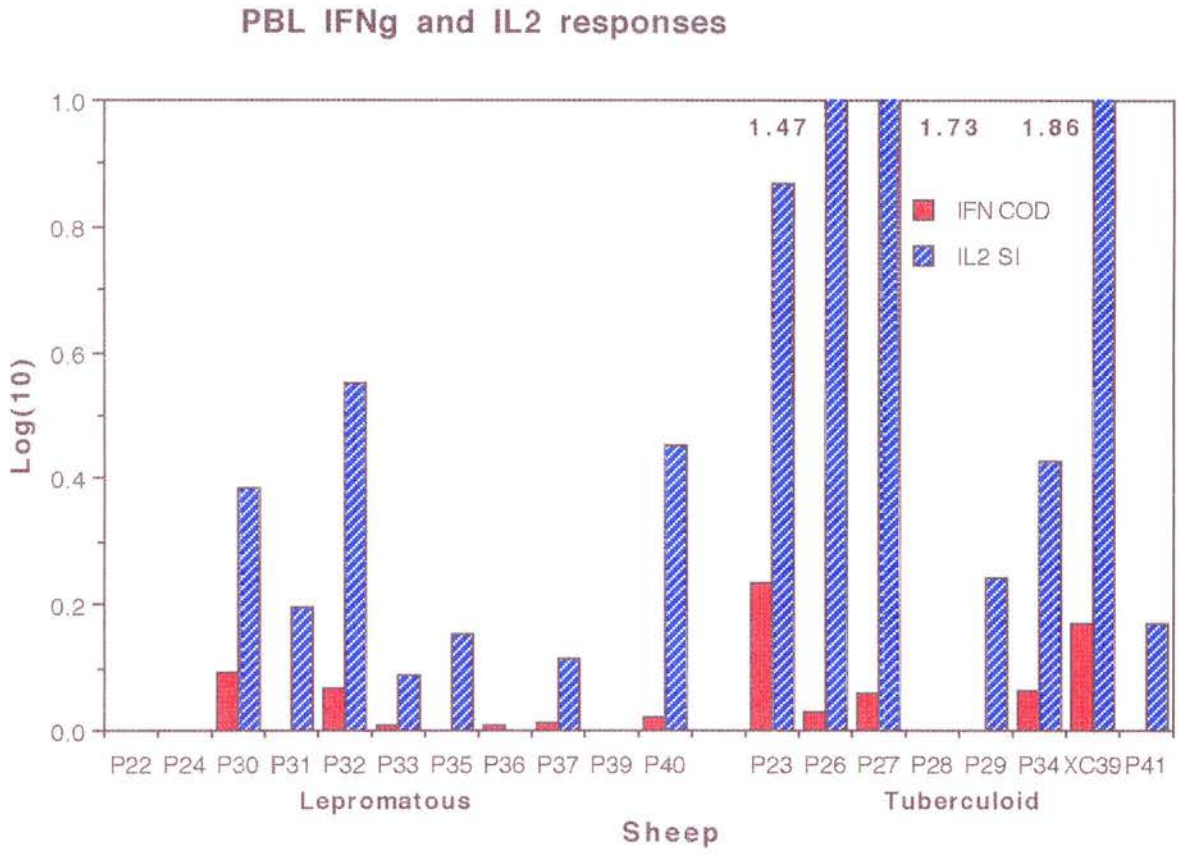


Figure 7.5: IFNγ and IL-2 elaboration by MLNL isolated from individual infected sheep of the lepromatous and tuberculoid groups.

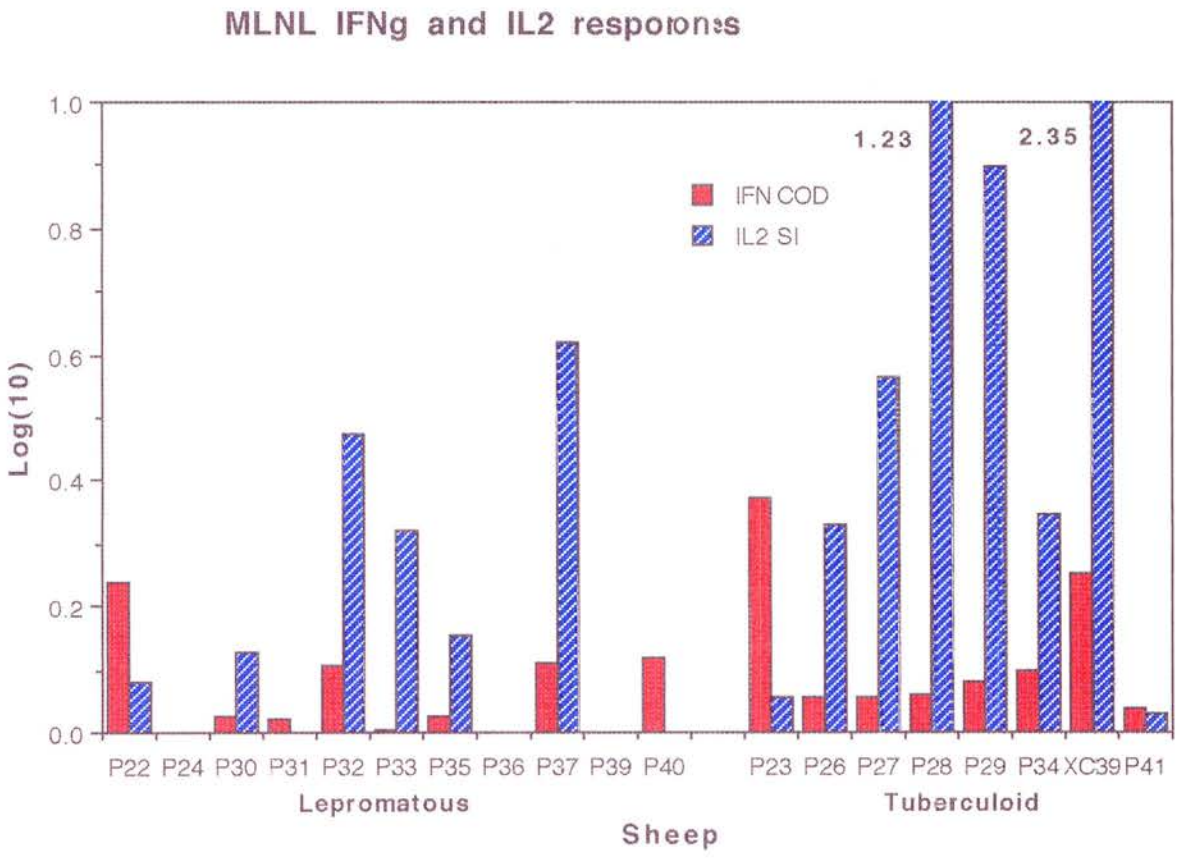
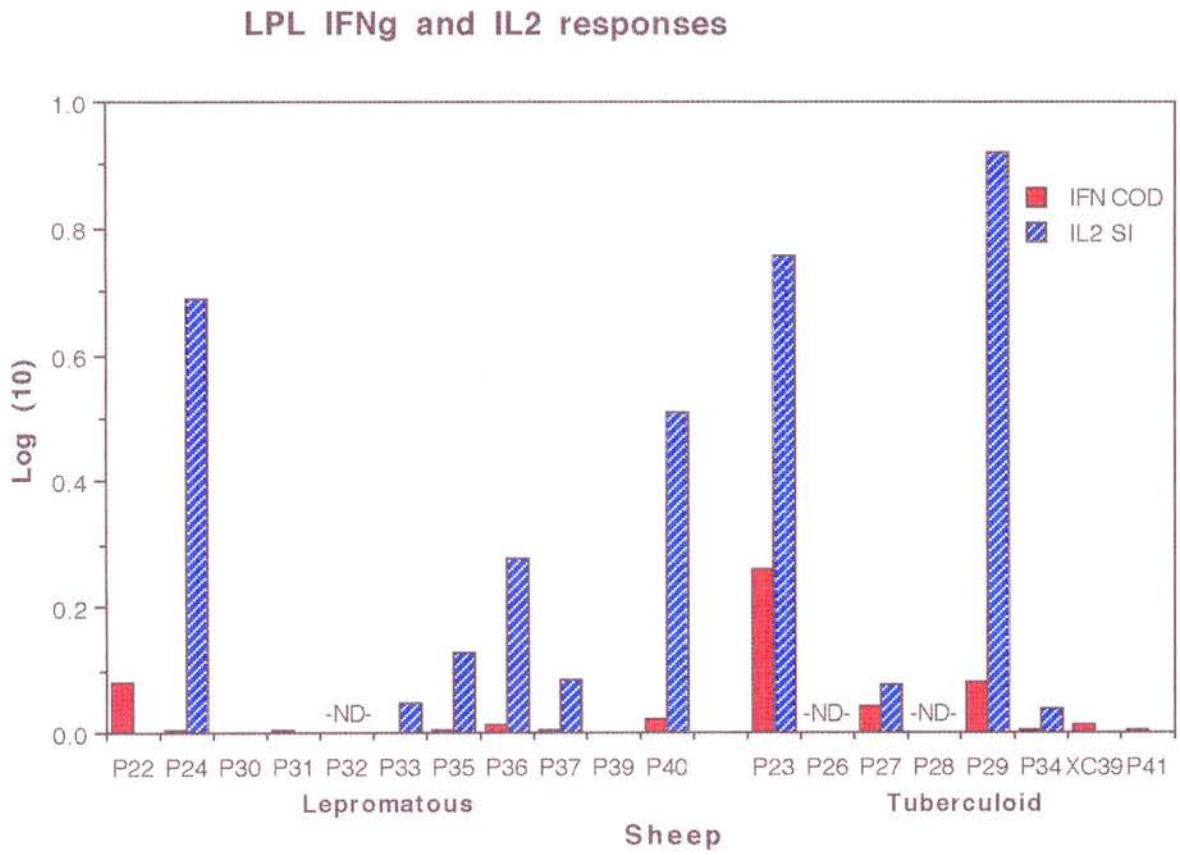


Figure 7.6: IFNγ and IL-2 elaboration by LPL isolated from individual infected sheep of the lepromatous and tuberculoid groups. ND: not determined.



animals of the tuberculoid group had measurable IFNγ production by MLNL, with four of these animals (50%) having in addition a positive IL-2 response (P23, P26, P27, XC39). Of the control animals, only two (13%) were positive for IFNγ (C43, C46), and one (6%) for IL-2 production (C43) (figure 7.2).

LPL were isolated and assayed from ten sheep of the lepromatous group, of which two (20%, P24, P40) were noted to produce positive levels of IL-2, with one sheep (10%, P22) producing IFNγ (figure 7.6). Six of the eight tuberculoid cases were examined for cytokine production by LPL, and of these three animals (P23, P27, P29) had positive IFNγ levels (50%), and of these three, two in addition had positive IL-2 values (33%, P23 and P29). None of the fourteen control animals had positive levels of IFNγ and only two (C41 and C47) had positive values for IL-2 production (14%) (figure 7.3).

7.3.2 Observations on groups of animals and median values

The median values for IFNγ COD and IL-2 SI are given in tables 7.2 and 7.3 respectively. Tables 7.4 and 7.5 show the geometric mean and 95% confidence intervals calculated from the IFNγ and IL-2 data. Figures 7.7 and 7.8 show the median values for each group of animals for IFNγ and IL-2 respectively.

Highest levels of both cytokines (as expressed in the median values) were noted in the tuberculoid group for all three tissues, with the exception of the median IL-2 value for LPL, which was slightly lower than that of the lepromatous group. Interestingly, however, both the minimum and maximum values of this parameter were higher for the tuberculoid than the lepromatous group, and this seemingly disparate value was "corrected" on the calculation of the geometric mean.

Table 7.1: Percentages of animals in each group which had positive levels of IFNγ and IL-2 production.

Group	*Control*		*Lepromatous*		*Tuberculoid*	
	IFN-γ	IL-2	IFN-γ	IL-2	IFN-γ	IL-2
PBL	19	31	19	6	63	50
MLNL	13	6	64	6	100	50
LPL	0	14	10	20	50	33

Table 7.2: Median values and ranges for IFNγ COD for all three groups of animals, and all three tissues examined. Significant difference from the control group is denoted *** (P<0.001)

Group	*Control*	*Lepromatous*	*Tuberculoid*
PBL	0.01 (0,0.36)	0.016 (0,0.167)	0.111 (0,0.71)
MLNL	0.014 (0,0.926)	0.058 (0,0.311)	0.174 (0.088,1.35)***
LPL	0.013 (0,0.039)	0.007 (0,0.046)	0.067 (0.005,0.82)

Table 7.3: Median values and ranges for IL-2 SI for all three groups of animals, and all three tissues examined. Significant differences from the control group are denoted * (P<0.05) and *** (P<0.001), and differences between the infected groups by # (P<0.05).

Group	*Control*	*Lepromatous*	*Tuberculoid*
PBL	2.05 (0.79,28.52)	1.31 (0.8,3.57)	5.03 (0.84,72.4)#
MLNL	1.23 (0.42,4.07)	1.21 (0.5,4.15)	2.95 (1.07,226.4)*#
LPL	1.08 (0.35,3.87)	1.17 (0.38,3.23)	1.14 (0.82,8.32)

Table 7.4: Geometric mean values and 95% confidence intervals for IFNγ COD for all three groups of animals, and all three tissues examined.

Group	*Control*	*Lepromatous*	*Tuberculoid*
PBL	0.045 (-0.002,0.095)	0.046 (-0.003,0.098)	0.175 (-0.006,0.39)
MLNL	0.063 (-0.025,0.158)	0.147 (0.019,0.29)	0.337 (0.061,0.685)
LPL	0.015 (0.008,0.022)	0.030 (-0.012,0.074)	0.166 (-0.079,0.483)

Table 7.5: Geometric mean values and 95% confidence intervals for IL-2 SI for all three groups of animals, and all three tissues examined.

Group	*Control*	*Lepromatous*	*Tuberculoid*
PBL	2.34 (1.39,3.95)	1.43 (1.01,2.02)	6.87 (1.59,29.72)
MLNL	1.21 (0.93,1.57)	1.19 (0.73,1.94)	5.32 (1.20,23.66)
LPL	1.11 (0.73,1.70)	1.23 (0.71,2.12)	1.91 (0.66,5.51)

Figure 7.7: Median IFNγ values for all three groups of animals, and for all three tissues examined. Significant difference from the control group is denoted *** (P<0.001).

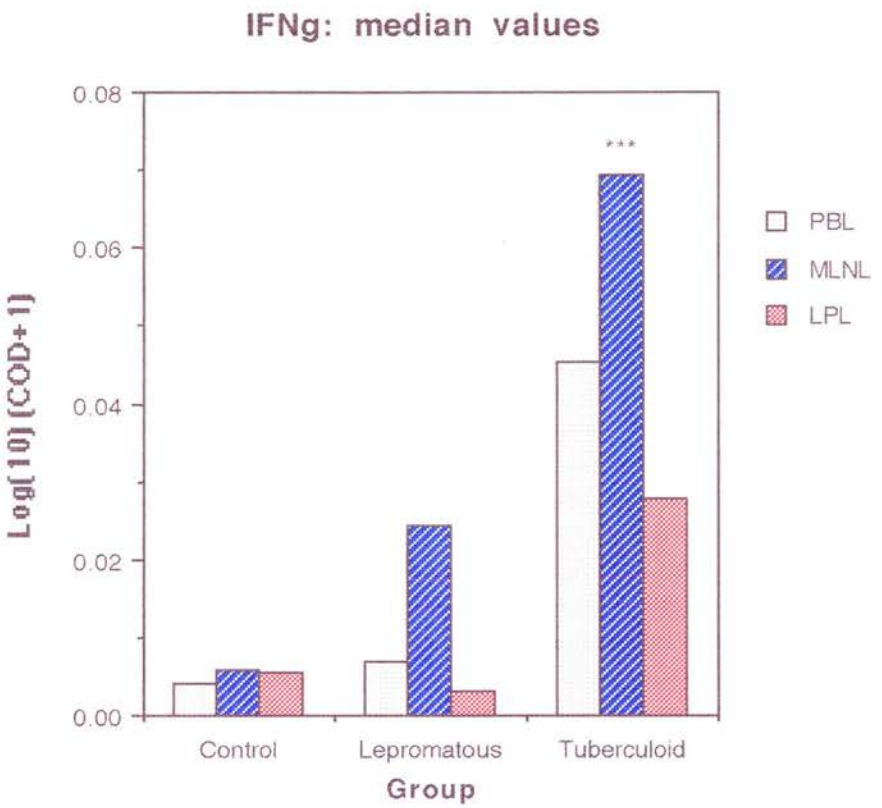
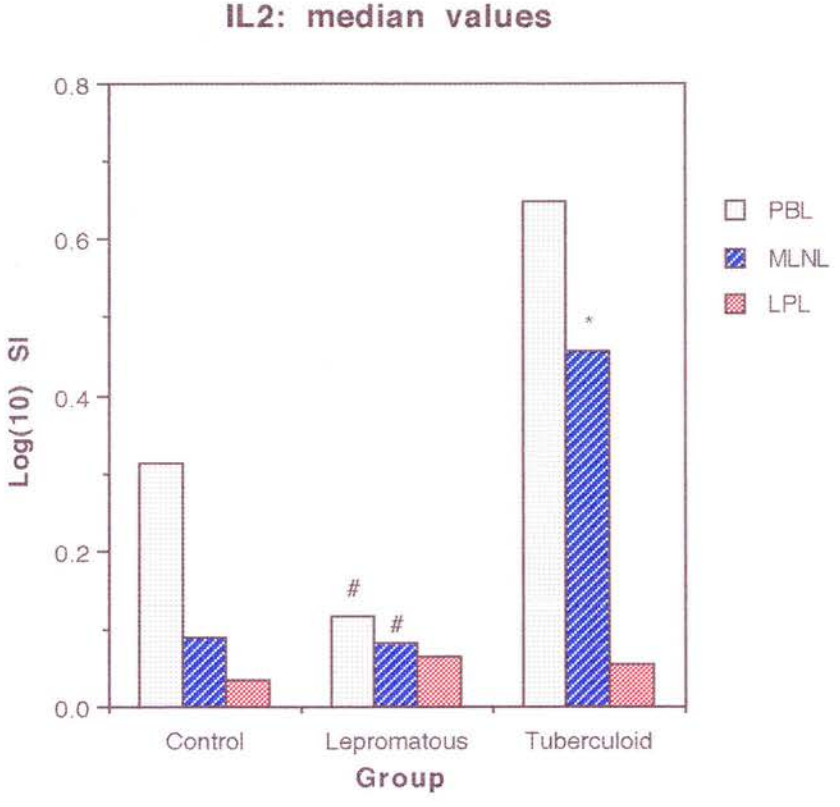


Figure 7.8: Median IL-2 values for all three groups of animals, and for all three tissues examined. Significant difference from the control group is denoted * (P<0.05), and between the lepromatous and tuberculoid groups by # (P<0.05).



On statistical examination, the tuberculoid group was found to have significantly higher levels of IL-2 than the control group for MLNL, and than the lepromatous group for both MLNL and PBL. IFNγ production by MLNL of the tuberculoid group was found to be greater than that of the control group ($P<0.001$). No significant differences were noted between the levels of cytokines produced by cells isolated from the control and lepromatous groups.

Of the three tissues examined, highest median levels of IFNγ were observed in the MLNL for each group of animals, followed by PBL (in both infected groups). In contrast, highest levels of IL-2 elaboration were noted by PBL, and then by MLNL for all three groups of animals. Low levels of IFNγ and IL-2 production by LPL were observed, and this was in keeping with the trend of low proliferative responses expressed in the LSA data in chapter 5.

7.3.3 Correlations

Spearman's Ranked Correlation calculations were performed using all nineteen infected animals as a single group, and included cytokine elaboration, and related this to LSA data, and also serum antibody ELISA data (both presented in chapter 5). A modest, positive correlation (0.627) was found between PBL IFNγ and PBL IL-2 production ($P<0.01$). Significant positive correlations ($P<0.05$) were also noted between PBL IFNγ COD and PBL LSA (weak correlation, 0.479), between PBL IFNγ COD and MLNL LSA (modest, 0.504), between PBL LSA and LPL IL-2 SI (modest, 0.562), and between PBL IL-2 SI and MLNL IFNγ COD (weak, 0.492). In addition, positive correlations were noted between LSA and IFNγ COD of MLNL (weak, 0.467), between LSA and IFNγ production of LPL (modest, 0.594), and between IFNγ production by LPL and MLNL (modest, 0.509).

When the tuberculoid group was examined independently of the lepromatous group, positive correlations were noted between LSA and IFNγ production by PBL (strong, 0.826), and between IL-2 and IFNγ production by LPL (strong, 0.886). For the lepromatous group alone, a positive correlation was found between IFNγ production by PBL and LSA of MLNL (modest, 0.667).

In animals of the control group, modest positive correlations were noted between IFNγ and IL-2 production by MLNL (0.654, $P<0.01$), and between LSA and IFNγ production by LPL (0.645, $P<0.05$).

7.4 DISCUSSION

The highest levels of IFNγ and IL-2 production were recorded in the group of animals which exhibited tuberculoid type pathology. Median IFNγ levels were seen to be higher in this group than in both the lepromatous and control groups, and on statistical examination IFNγ production by MLNL from tuberculoid cases was found to be significantly higher than that of the control group. Likewise, IL-2 production was significantly higher in MLNL from animals with tuberculoid lesions than both lepromatous and control animals. Similarly, PBL from the tuberculoid group produced a significantly higher median IL-2 value than that of the lepromatous group.

It is interesting that no significant differences were noted in the median values of the lepromatous and control groups. This suggests that there exists very little antigen-specific DTH type reaction in the cases with lepromatous disease, as measured by these type 1 cytokines, and would appear to be in keeping with the observed low *in-vivo* DTH as measured by the intra-dermal Johnin test in cattle with lepromatous disease (Bendixen, 1978; Chiodini *et al.,* 1984). Some individual animals of the lepromatous group had positive levels of IFNγ and of IL-2 activity, but the majority

had very low levels of these two cytokines. However, measurement of type 2 cytokines was not attempted in this study, due to the lack of availability of specific assays for these cytokines in the sheep. Likewise, other mechanisms of suppression of DTH are possible, as discussed in chapter 5, such as factors of mycobacterial origin.

IFNγ production was highest in cells isolated from MLN, and this was likely to be a reflection of the numbers of antigen-specific lymphocytes in this tissue which drains the primary site of infection and lesions. It may be expected that there is a relatively lower percentage of antigen-specific cells in the peripheral circulation. Indeed it is also possible that cells of the lamina propria may be under the influence of what is considered to be a type 2 environment, where a considerable part of the immunological function of this organ is the production of antibody at a mucosal surface. It may be in the interests of the host to avoid DTH reaction and thereby maintain the normal architecture and hence digestive functions of the gut. There have, however, been conflicting reports on IL-2 activity and expression of IL-2 mRNA in the gut of human patients with inflammatory bowel disease (IBD), with some workers reporting increased levels in the lesions of IBD (Mullin *et al.,* 1991), and others a decrease (Kusugami *et al.,* 1991). It has been suggested that LPL in particular proliferate and elaborate cytokines in response to stimulation of the CD2 pathway, instead of through the CD3-TCR complex (Targan *et al.,* 1995), and this may explain the relatively low proliferative and cytokine responses of these cells in this study.

Conversely, IL-2 production was highest in PBL, followed by MLNL. The one difference in the assay for cytokines of PBL origin is that whole blood culture was performed, whereas the other MLNL and LPL cells were performed on isolated, Lymphoprep purified, and washed cells. It is possible that some residual endogenous IL-2 and/or IFNγ could have existed in the plasma in the whole blood cultures, or that

the cellular population assayed was not the same due to the absence of the Lymphoprep purification and washing steps included in the isolation of MLNL and LPL. In retrospect, all cell populations assayed should have been subjected to the same purification protocols, as was the case for the proliferation assays in chapter 5. The same cascade of events is involved in the production of IFNγ and IL-2 as for cellular proliferation, with T lymphocyte stimulation requiring antigen presentation by APCs and secondary costimulatory signals, resulting in the formation of antigen-specific T-cell clones which produce these cytokines as effector mechanisms in the responses to the pathogen. Therefore the same observations on the potential differences in the cell populations in the cell suspensions assayed as discussed in chapter 5 are pertinent in this situation also. Variations in the numbers of both antigen-specific memory T cells and APCs therefore would result in differing levels of cytokine production for each tissue assayed *in-vitro*.

The use of the IFNγ ELISA has been proposed and performed as a diagnostic tool for mycobacterial diseases of livestock, in particular for tuberculosis in cattle (Rothel *et al.,* 1990; Wood *et al.,* 1991 and 1992) and has since been modified for the detection of paratuberculosis in cattle (Billman-Jacobe *et al.,* 1992) and sheep (Pérez *et al.,* 1994). On the basis of the results of this study, the use of this assay in the diagnosis of ovine paratuberculosis may be useful in cases with tuberculoid lesions, or in the diagnosis of early infections as described by Pérez *et al.* (1994), where CMI is likely to be dominant and the evaluation of serum antibody is unlikely to be helpful due to low levels of humoral immunity. It appears that cases with lepromatous lesions may not be consistently diagnosed using whole blood IFNγ ELISA.

In general, more individual infected animals were found to produce IFNγ than to produce IL-2, with the sole exception of the LPL of the lepromatous group, in which two animals produced IL-2, whereas only one animal had a positive level of IFNγ

production. This may be a result of higher sensitivity of this test (Burrells *et al.*, 1995), which relies on direct measurement of captured cytokine, and not on its biological activity in a bioassay.

These results suggest a clear relationship between the histological lesions and production of the cytokines IFNγ and IL-2, with lymphocytes isolated from the circulation and the local tissues of animals with tuberculoid pathology showing higher levels of these cytokines than those from animals with lepromatous lesions. Dominance of these cytokines in the tuberculoid group suggest strong DTH responses associated with tuberculoid pathology, and a relatively poor DTH in the animals with lepromatous stage of the disease. Several significant correlations were noted between cytokine elaboration and cellular proliferation across the range of tissue samples and in all three groups of sheep. Higher levels of IFNγ and IL-2 suggest that type 1 cells may be important in the formation of tuberculoid lesions, and conversely, that cells of this type may be less involved in the lepromatous cases. This would be in keeping with the high numbers, persistence and continued growth of intracellular mycobacteria in this group. Because of the lower levels of type 1 cytokine production by lymphocytes isolated from lepromatous cases, it may be possible that a type 2 response is dominant in animals of this group, although it was not possible to assay type 2 cytokines in this study.

Studies in other mycobacterial diseases, and also in diseases such as listeriosis and leishmaniasis, which are a result of intracellular infections, have provided evidence that Th1 type cytokines are associated with DTH and protective or strong immunity, whereas dominance of type 2 cytokines equates to susceptibility and diffuse disease. Examination of cytokine elaboration by human T-cell clones (both from healthy and diseased patients) in response to mycobacterial antigens has been performed by a number of workers. Mycobacterium-reactive $CD4^+$ T-cell clones from

healthy human subjects have been shown to preferentially produce IFNγ and IL-2 (Del Prete *et al.,* 1991; Haanen *et al.,* 1991). *M. leprae*-specific $CD4^+$ T-cell clones from the PBL and lesions of patients with tuberculoid leprosy were found to produce high levels of IFNγ but little or no IL-4, IL-5 or IL-6, whereas *M. leprae*-specific $CD8^+$ clones isolated from PBL and lesions of patients with the lepromatous form of the disease elaborated lower levels of IFNγ, and high levels of IL-4 and IL-5. The functions of these cells were suggested to be DTH and suppressor cells respectively (Salgame *et al.,* 1991). In a similar study, Mutis *et al.* (1993) described high levels of IFNγ and TNFα secretion by T-cell clones generated from PBL of donors with tuberculoid leprosy lesions. These observations on cytokine production have been corroborated by studies of cytokine mRNA expression in tissue biopsies in which polar forms of leprosy (Yamamura *et al.,* 1991) and reversal reactions (Yamamura *et al.,* 1992) were examined. In the biopsies of tuberculoid lesions, reversal reactions and lepromin tests, it was found that mRNA for IFNγ, IL-2 and TNFβ were dominant, whereas lepromatous lesions and those of erythema nodosum leprosum (ENL) were associated with high levels of IL-4, IL-5 and IL-10.

A study of experimental murine *M. tuberculosis* infection described dominant Th1-like, IFNγ-secreting $CD4^+$ lymphocytes in the early stages of infection which correlated with protective immunity. However, as infection progressed IL-4 was increasingly secreted and became dominant (Orme *et al.,* 1993). In human tuberculosis, Barnes *et al.* (1993b) evaluated IFNγ and IL-2 mRNA levels in cells isolated from the pleural fluid of patients with tuberculous pleuritis, and also production of IFNγ and IL-2 from cell culture supernatants. PCR products for IFNγ and IL-2 were markedly increased in pleural fluid, and the concentration of IFNγ was significantly greater in cell culture supernatants when compared with PBL. On the other hand, Barnes *et al.* (1993a) examined cytokine production by PBL-derived, *M. tuberculosis*-specific, human αβ- and γδ TCR^+ T-cell clones and found that these

cells elaborated a wider, and less definable array of cytokines. In the latter study, clones were most frequently found to produce IFNγ, TNFα and IL-10, a pattern which resembles that described for murine Th0 cells. Interestingly, the γδ TCR^+ clones were observed to produce very high levels of TNFα, a cytokine which is considered to be important in the regulation of granuloma formation (Kindler *et al.,* 1989). Elevated TNFα mRNA expression was found in the ileal mucosa of ovine paratuberculosis cases, both in sheep with lepromatous and tuberculoid pathology, and in addition TNFα activity was noted in supernatants from in-vitro intestinal culture (Alzuherri *et al.,* 1996).

A clear pattern has therefore been established in other mycobacterial infections whereby high immunity, resistant states are characterised by dominance of type 1 cytokines, and multibacillary, susceptible cases by dominance of type 2 cytokines. On the basis of the results of IFNγ and IL-2 production in this study, it appears that a similar pattern is present in *Map* infection in sheep, with tuberculoid, paucibacillary lesions associated with dominance of type 1 cytokines, particularly in the local tissues, as reflected in the results of the MLN cells, where all animals produced IFNγ in response to antigenic stimulation. Production of these cytokines appears to correlate with cellular proliferation in response to *Map* antigen, and is most evident in sheep with tuberculoid lesions.

CHAPTER EIGHT

MOLECULAR INVESTIGATION OF CYTOKINE mRNA LEVELS IN PARATUBERCULOSIS

8.1 INTRODUCTION

Assays for ovine IFNγ and IL-2 only were available, and elaboration of these cytokines in response to antigenic stimulation of LPL isolated from the lesions of paratuberculosis proved to be of relatively low magnitude. Therefore, in order to further examine the production of these and other cytokines, PCR technology was chosen for a preliminary study of cytokine levels in the lesions of paratuberculosis. Examination of levels of cytokine messenger RNA (mRNA) in the tissues of infected and normal, control sheep was possible by the use of RT-PCR techniques, and in addition permitted determination of type 2 cytokines IL-4 and IL-10, for which there were no assays available.

PCR is an *in vitro* DNA amplification technique which consists of a repeated cycling process involving three distinct stages (Rapley *et al.,* 1992). Firstly, double-standed sample DNA is denatured into two complementary single-strand molecules by heating to 95°C. This is followed by the annealing stage, in which the temperature is reduced to allow binding of oligonucleotide primers to their complementary single-strand DNA sequences. The final stage is an extension reaction, in which thermostable DNA polymerase synthesises a complementary copy of the initial single strand by extension of the 3' end of the annealed primer. PCR products accumulate exponentially as the reaction progresses. Five main components are required for the PCR to proceed: target DNA template onto which annealing and synthesis can take place; a pair of oligonucleotide primers, sense and antisense; *Taq* polymerase; the four

deoxynucleotide triphosphate (dNTP) 'building blocks' in equimolar concentrations; and finally a PCR buffer, containing $MgCl_2$ in particular, in order to optimise *Taq* polymerase action. PCR is a sensitive technique by which, in theory, a single target DNA molecule can be amplified and thus detected. RT-PCR method is based on the ability of reverse transcriptase enzyme to transcribe the relatively labile RNA molecule into a more stable complementary DNA (cDNA) sequence.

RT-PCR technology was employed in order to assess the expression of cytokine mRNA in the ileum. The PCR products were subsequently Southern blotted and probed using radiolabelled internal sequence oligonucleotide probes in order to further increase sensitivity and to show specificity. The cytokines selected for investigation were those that had previously been shown to be important in both the induction and suppression of DTH responses, namely IFNγ, IL-4 and IL-10. In order to achieve an assessment of IL-2 activity in the lesions, the expression of mRNA for the IL-2 receptor (IL-2r) was examined, as this receptor molecule (CD25) is expressed on T cells in response to IL-2-mediated T-cell activation (Bujdoso *et al.,* 1992 and 1993). Expression of mRNA for the type 2 cytokines IL-4 and IL-10 was investigated, as they have been considered to exert a suppressive effect on DTH, and on type-1 cytokine activity, and it has been suggested that type 2 cytokines are dominant in lepromatous lesions of leprosy (Modlin, 1994). In addition, DNA extraction and subsequent PCR investigation was performed to evaluate the presence of *Map* -specific IS900 DNA sequences (Vary *et al.,* 1990) in ileal samples of sheep with clinical paratuberculosis.

In order to verify that RNA isolation and reverse transcription had been performed successfully, the presence of mRNA for ATPase, a marker for all cells, was initially assayed by PCR (Woodall *et al.,* 1994). Samples were then subjected to PCR for specific cytokine messages. This study was qualitative in nature, and was

intended to provide preliminary data on the presence of cytokine mRNA in the ileum in paratuberculosis.

8.2 MATERIALS AND METHODS

8.2.1 Collection of ileal samples for molecular investigation

Tissues were collected fresh at necropsy and snap frozen in isopentane/CO_2 slush as described in chapter 3, without the embedding of tissue in OCT compound, and were stored in plastic sachets at -70°C until required. Care was taken to maintain all samples at -70°C, as elevation of sample temperature may permit degradation of mRNA. Tissues from twenty infected sheep (ten sheep of the lepromatous and ten of the tuberculoid groups) and ten normal control sheep were subjected to molecular investigation for cytokine mRNA, and these sheep are listed in the following table.

Control	Lepromatous	Tuberculoid
C32	P18	P19
C33	P21	P23
C36	P24	P25
C37	P30	P27
C38	P31	P29
C41	P35	P34
C42	P36	XC39
C43	P37	P41
C44	P39	P42
C45	P46	P49

8.2.2 Extraction of genomic DNA from ileum

In order to look for the presence of *Map*-specific DNA sequences, DNA was extracted from ileum using a commercially available genomic DNA extraction kit (InViSorb Genomic DNA kit, Bioline (UK) Ltd., London). DNA extraction was

performed according to the manufacturer's instruction manual, using a sample of ileum of approximately 10 mg in weight. In brief, a 10 mg piece of ileum was placed into an Eppendorff tube containing 500 µl of Lysis Buffer and thoroughly macerated using a glass rod, and subsequently incubated at room temperature for 30 minutes. The tube was then centrifuged briefly to pellet particulate material and the supernatant removed to a fresh Eppendorff tube, to which 15 µl of Carrier Suspension was added and the tube vortexed. The tube was then placed on ice for 5 minutes before centrifuging in a microcentrifuge at 13000 rpm for 20 seconds, and the supernatant then discarded. The pellet was washed three times by resuspending in 1 ml Wash Buffer and then centrifuging for 15 seconds. The resultant pellet was then dried by incubating in a water bath at 52°C to evaporate residual wash buffer. DNA was then eluted by adding 50 µl Elution Buffer and vortexing, followed by incubation at 52°C for 5 minutes. DNA was then harvested by centrifugation of the tube for 5 minutes at 13000 rpm and pipetting off the resultant supernatant, which was stored at -20°C until required for PCR.

8.2.3 RNA extraction from ileum

The isolation of RNA is complicated by the fact that RNA is a relatively unstable molecule which is readily degraded by ribonuclease (RNase) enzymes which are ubiquitous in the environment, and in particular are present on the skin of the handler. PCR is a sensitive technique and very small amounts of contaminant DNA can be readily amplified in the reaction. In order to minimise these complications, care was taken to avoid the introduction of RNase and contaminant DNA to the samples, solutions and laboratory ware. RNA isolation and handling was performed in a laminar flow hood. Disposable latex gloves were worn at all times and were changed frequently. All equipment was thoroughly cleaned and sterilised using 70% ethanol

before and after use. Sterile, disposable plasticware and sterile, RNase-free, plugged pipette tips (Biorad Labs, Hercules, USA) were used for all samples. RNase-free water was prepared by DEPC treatment. In order to reduce the likelihood of sample contamination with PCR products, RNA isolation and cDNA synthesis were performed in a distant laboratory from that in which the PCR was run and PCR products subsequently handled and stored.

RNA extraction from ileal samples was performed using the commercially available 'RNEasy' Total RNA Extraction Kit (Qiagen Inc., Chatsworth, USA). The protocol for use was supplied with the kit and was in general adhered to with some exceptions, due to the difficulties encountered in the handling of gut which contains large amounts of mucus. Fresh working solutions were made up before each extraction. Lysis buffer was prepared for use by the addition of 10 µl 2-mercaptoethanol per ml of lysis buffer ('RLT') stock-solution. A working dilution of wash buffer ('RPE') was prepared by addition of 4 parts 100% ethanol to 1 part wash buffer concentrate. A 1400 µl aliquot of lysis buffer was placed in a sterilised, glass tissue-homogeniser which had been pre-chilled on ice. The tissue sample was removed from -70°C storage, and a 30 mg sample of this was immediately placed into the lysis buffer and homogenised until the sample was thoroughly disrupted. The recommended volume of lysis buffer stipulated in the manufacturers instructions was 600 µl, but this was found to result in a highly mucoid solution and as such was difficult to handle. Therefore the optimal volume was found to be 1400 µl. The homogenate was decanted into an Eppendorff 1.5 ml microcentrifuge tube and centrifuged at 15000 x g (13000 rpm) for 15 minutes to pellet tissue fragments and mucus. The supernatant was collected into a new 1.5 ml tube and mixed with an equal volume of 70% ethanol, in order to precipitate the RNA, and the resultant mixture was pipetted onto the RNEasy column, which was then centrifuged for 15 seconds at 10000 x g. The flow-through was discarded, 700µl of wash buffer 'RW1' was

pipetted onto the column, and the column was re-centrifuged at 10000 x g for 15 seconds. A new 2 ml collection tube was applied to the column, before 500 µl of wash buffer 'RPE' (working dilution) was pipetted onto the column, which was recentrifuged at 10000 x g for 15 seconds. This wash was then repeated, and the column dried by centrifugation for 2 minutes. An Eppendorff 1.5 ml collection tube was then fitted to the column, and 40 µl of RNase-free, sterile, distilled, tissue culture water (Sigma) ,was pipetted onto the column which was then incubated on ice for 5 minutes. The column was then subjected to centrifugation at 10000 x g for 60 seconds to elute the RNA. The flow-through solution containing the extracted RNA was stored at -70°C until used for reverse transcription to cDNA.

8.2.4 Gel electrophoresis of RNA

The presence of RNA was verified by electrophoresis through a 2% agarose minigel and visualised using ethidium bromide. The gel was run in a TAE buffer, which was made up as a x50 concentrated solution (appendix 8.1). A working solution was made by adding 10 ml of this stock solution to 490 ml of distilled water prior to use. 1 g of agarose (Sigma) was added to a sterile bottle containing 50 ml of TAE buffer and dissolved by heating in a microwave oven. The agarose solution was cooled to approximately 50°C before 2.5 µl of ethidium bromide was added and mixed into the solution. The gel was then poured into a ethanol-cleaned gel-trough, and left at 4°C until the gel had set. 22.5 µl of ethidium bromide was added to the remaining 450 ml of the diluted TAE running buffer which was added to the electrophoresis apparatus. RNA was denatured to single strand before gel electrophoresis. A 5 µl aliquot of the RNA sample was therefore mixed with 3 µl loading buffer (Sigma) and heated at 80°C for 3 minutes and subsequently cooled in a freezer at -20°C for a further 5 minutes. The samples were then added to the wells in the submerged gel and

the gel run at 50 mA for approximately 30 minutes until the coloured sample front had migrated approximately half way along the gel. The gel was then viewed using ultraviolet transillumination and photographed to check for the presence of RNA.

8.2.5 Reverse transcription of RNA to cDNA

For each RNA sample, duplicate tubes of cDNA were prepared and these were pooled at the end of the reaction. Fresh reverse transcription mix was prepared by mixing multiples of the following amounts of reagents for each sample: 4 µl x5 Superscript buffer (Gibco BRL), 2 µl random hexamer primers (R6MERS) (Pharmacia) which had been pre-prepared according to the manufacturers instructions, 1 µl 20nM dNTPs (Pharmacia), and 2 µl DTT (Gibco). For each sample, 9 µl of this mix was placed in a 0.5 ml Eppendorff tube, and 10 µl of RNA sample added. The RNA was then denatured to single strand by heating this mixture to 80°C for 3 minutes on a heated block, and then cooled for 5 minutes on ice. One µl Superscript reverse transcriptase (Gibco) was then added to each tube. Care was taken to maintain the temperature of the Superscript enzyme below 0°C at all times. The tubes were incubated for 60 minutes in a water bath maintained at 45°C until the reverse-transcription reaction was terminated by incubating the tubes for 5 minutes at 95°C in a heated block. Thereafter, the tubes were immediately cooled on ice for 5 minutes and the cDNA then stored at -20°C until required for the PCR reaction.

8.2.6 PCR protocol

Working on ice, the PCR mix was prepared by mixing multiples of the following reagent quantities for each sample in an Eppendorff tube: 5 µl x10 *Taq*

buffer (Gibco), 2.5 µl 50mM $MgCl_2$ solution (Gibco), 1 µl W1 detergent (Gibco), 1 µl 20mM dNTPs (Pharmacia) and 37.5 µl RNA water (Sigma). To this mix, 0.5 µl each of primers 1 and 2 were added per sample (25pmol l^{-1}). 46 µl of this mix was then added to each labelled 0.5 ml Eppendorff tube which had been pre-cooled on ice. The resultant mixtures were 'sealed' by the addition of one drop of mineral oil (Sigma) to each tube. A 4 µl aliquot of each individual DNA sample was then pipetted into each sample tube through the oil layer, and the tubes then transported on ice to the distant PCR laboratory. On arrival, 4 µl RNA water was added to the negative control, and 1 µl of known positive PCR product to the positive control tubes. The tubes were placed in the dry heating block of the air heated/cooled PCR machine (Techne PHC1) where they were heated at 95°C for 5 minutes, after which the temperature was rapidly reduced and maintained at 80°C. A 0.2 µl volume of *Taq* DNA polymerase (Gibco BRL) was then added to each sample beneath the oil layer. The samples were then thermally cycled in the PCR thermal cycler machine using a programme that had been optimised both for temperature and number of cycles for the primers used. The protocol typically consisted of 30-33 cycles of denaturation at 95°C for 45 seconds, an annealing phase of variable temperature for 45 seconds, and an extension phase of 72°C for 2 minutes, followed by a final extension phase of 72°C for 5 minutes. Details for individual primers are included in table 8.1.

8.2.7 Agarose gel electrophoresis of PCR product

Agarose gel electrophoresis was performed for the separation and examination of PCR products. Agarose gel (2%) was prepared as described above (section 8.2.4) except using a larger gel apparatus and therefore using 6 g agarose in 300 ml of TAE buffer, with 15 µl of ethidium bromide added, and the gel submerged in 1700 ml TAE buffer containing 89 µl ethidium bromide. Care was taken to wipe mineral oil from the

Table 8.1: Primers and probes, including sequence (listed as 5' to 3' in the order of sense, antisense, probe), optimal annealing temperatures and number of cycles, and the sizes of the PCR products.

Specificity	*Primer*	*Sequence*	*Optimal annealing*	*No. cycles*	*Product size bp*
ATPase	2a+	GCT GAC TTG GTC ATC TGC	55°C	33	165
	2a-	CAG GTA GGT TTG AGG GGA TAC			
		CAT CCC CTG CTG GAA GAC GGA ATT			
IFNγ	H0903	TGA AAT ACA CAA GCT CCT TC	50°C	32	416
	H0904	TCA CCT TGA TGA GTT CAT TGA			
		CAA GAC ATG TTT CAG AAG TTC TTG AAC GG			
IL-2r	G2203	ACC TTC CAG GTC ACT GCG AGG	61°C	30	385
	G2204	CTG CGA TCT GGT ACT CGG TGG			
		GGG CAG ACG GTT CAC TAC CAG TG			
IL-4	H0900	TGA TCC CAG CGC TGG TGT GC	57°C	32	338
	H0901	GTC TCT CAG CGT ACT TGT ACT			
		AGC CAC ATG TGC TTG AAC AAA TTC CT			
IL-10	E419	ATG CCA CAG GCT GAG AAC C	54°C	31	452
	860X	GTT CAC AGA GAA GCT CAG T			
		ACC TGC TCC ACC GCC TTG			
IS900	1932	GCG GGA TCC GTG GCA CAA CCT GTC TG	55°C	33	1200
	1933	GCG GGA TCC TCA AGC CGC CGC GGT AG			

pipette tip, and 7 µl PCR product from each sample was added to 2 µl gel loading solution, mixed, and applied to the submerged gel. In order to assess the size of the PCR product, a 1 kb DNA ladder (Gibco) was also added to each gel before applying an electrical current of 50 mA and running for 2 hours, until the coloured sample was visible approximately half way across the gel. PCR products were then visualised using ultraviolet light transillumination and the gel photographed. The gel was then trimmed to size and measured in preparation for Southern blotting.

8.2.8 Southern blotting protocol

Gels were first washed by agitation in a 0.4M NaOH solution for 30 minutes on orbital shaker apparatus. A piece of 'Genescreen Plus' nylon hybridisation membrane (DuPont, Massachusetts, USA) was cut to the size of the gel, marked with pencil to allow confirmation of orientation, and 'equilibrated' by soaking in distilled water, to which was added the same volume of 0.4M NaOH solution, for 15 minutes at room temperature.

Southern blotting was performed in the gel apparatus that had been used to run the gel, and 'Whatman no.4' filter paper was cut to fit the gel tray with ends submersed in the gel tank troughs and a further four pieces were then cut to the exact size of the gel. The large, bottom piece of filter paper was placed in the gel tank and wet with 0.4M NaOH. Care was taken to remove any air-bubbles from under the filter paper. The washed gel was placed upside down on the filter-paper base, and the hybridisation membrane placed on the upturned gel. Two pieces of the cut filter paper were soaked in 0.4M NaOH and placed on top of the membrane, again taking care to exclude air-bubbles, and a strip of 'Nescofilm' was placed around the very edges of the gel to prevent inappropriate blotting. Two dry pieces of filter paper were then

added, followed by a stack of folded tissue paper to promote blotting through the gel and membrane by capillary action. A flat plate of glass was then placed on top of the stack and weighted-down evenly by putting a 1 kg weight on top of the glass. The gel-tank troughs were filled with 0.4M NaOH and the membrane left to blot for 16 hours at room temperature. After blotting was complete, the membrane was removed and washed twice in x2 SSPE buffer prepared by 1:10 dilution from x20 SSPE stock solution (appendix 8.1). The membrane was then placed on filter paper and crosslinked using an automatic programme in an ultraviolet crosslinking machine. The membrane was subsequently dried at room temperature, and stored in the dark in folded filter paper.

8.2.9 Radiolabelled oligonucleotide probing

The oligonucleotide specific for the internal sequence of the PCR product being probed was firstly radiolabelled with ^{32}P isotope using polynucleotide kinase enzyme (PNK) to transfer this molecule from an ATP molecule to the oligonucleotide (appendix 8.1). The unbound ATP molecules were then removed from the reaction mix using a sephadex column (Nick column, Pharmacia, Uppsala, Sweden). The column was first wet with TRIS-EDTA buffer at pH 7.4 (appendix 8.1), and the PNK reaction mix was then added and the radiolabelled oligonucleotide eluted by adding a further 400 µl of TRIS-EDTA buffer. The purified probe was then used in the hybridisation mix. Hybridisation buffer solutions for probing were freshly prepared (appendix 8.1).

The blotted membrane was placed in a cylinder in a hybridisation oven (Techne hybridiser HB-1) with 50 ml of x2 SSPE and rotated to remove bubbles. This solution was decanted and 20 ml of hybridisation buffer was added and the cylinder rotated for

30 minutes at 65°C. This solution was decanted and a further 10 ml of hybridisation solution added to the cylinder before 100 µl of the radiolabelled probe was added. The chamber was then left to rotate for 12 hours at 65°C. After hybridisation, the membrane was washed twice in 20 ml of x2 SSPE for 5 minutes at room temperature, and then washed twice with 30 ml of x2 SSPE+1%SDS for 20 minutes at 60°C. Finally the membrane was washed in x0.1 SSPE at room temperature for 20 minutes before being wrapped in PVC film and placed on an X-ray film (Kodak X-OMAT) in a film-cassette for a variable period dependent upon the level of radioactivity present on the membrane as measured by a Geiger-Muller counter. A period of 12 hours was normally adequate. The film was developed using an automated radiograph processor.

8.2.10 Examination and interpretation of autoradiographs

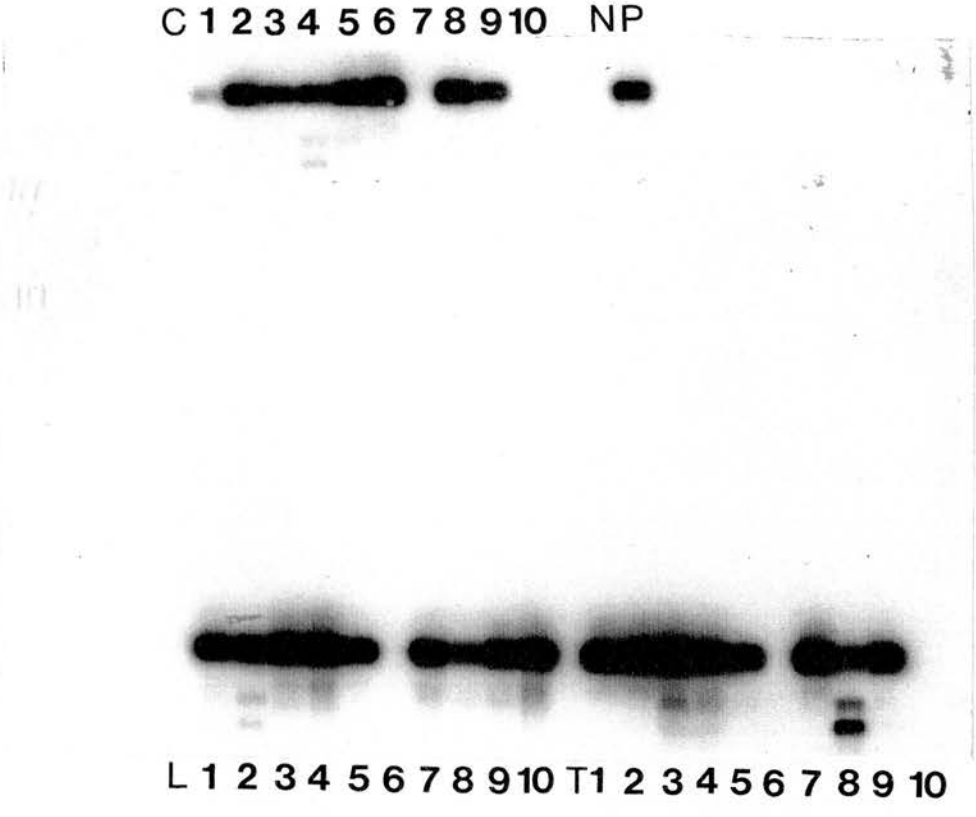
Autoradiographs were examined on an illuminated light box, and the intensity of the sample bands on each radiograph were assessed subjectively and scored on a scale of 0 to 5, based on absence of a visible positive band (scored 0) to a strong, highly dense, positive band (scored 5). Scores assigned to individual samples are included in table 8.2. These scores were then 'normalised' to those of the ATPase autoradiograph. This was performed by multiplication of the ATPase value of the individual cDNA sample by the appropriate constant to result in a score of 5, and thereafter mutiplying the values of the other scores for the same sample by the same constant. For example, an ATPase score of 3 resulted in all scores for that sample being multiplied by 5/3. Normalised data were subsequently subjected to Mann-Whitney testing using the Minitab for Windows statistical software package.

Table 8.2: Scores were assigned to sample bands on autoradiographs of PCR products on the basis of band intensity. Scores were allocated using a scale of 0-5, with 0 indicating absence of a visible band, and 5 indicating a band which appeared very strong. Sheep numbers are listed in the order of control, lepromatous and tuberculoid groups. Also included is the result of PCR examination for the presence of *Map*-specific IS-900, with positive samples denoted +.

Sheep	ATPase	IFNγ	IL-2r	IL-4	IL-10	IS-900
C32	3	0	0	0		
C33	5	2	0	0	1	
C36	4	3	2	2	2	
C37	4	0	0	0	2	
C38	5	1	2	0	2	
C41	0				0	
C42	5	3	3	1	2	
C43	0	3	2	2	2	
C44	5	3	2	2	1	
C45	4	2	1	0	1	
P18	5	3	3	2	3	+
P21	5	1	1	0	1	+
P24	5	4	3	3	4	
P30	5	4	5	2	4	+
P31	5	4	5	3	4	+
P35	0	4	3	2	5	+
P36	5	4	5	2	4	+
P37	4	4	4	2	4	+
P39	5	4	0			
P46	5	0	3	3	2	
P19	5	5	4	4	3	+
P23	5	5	4	2	3	+
P25	5	5	4	3	3	
P27	5	3	3	3	2	
P29	5	2	3	3	2	+
P34	0		0	3	2	+
XC39	5	3	3	2	3	
P41	4	3	0	0	1	+
P42	5	0	1	3	1	
P49	0	3	5	3	4	

Figure 8.1 (opposite): Photograph of autoradiograph of Southern blotted ATPase PCR products. Samples from control animals (C) are above, and those from animals of the lepromatous (L) and tuberculoid (T) groups are below, and sample-lane numbers correspond to individual animals as indicated below. N: negative-, and P: positive-control samples.
Positive bands were absent from four samples: C43 and C41 of the control group, P35 of the lepromatous group, and P34 and P49 of the tuberculoid group (lanes C7, C10, L6, T6 and T10 respectively).

Lane number	C: Control	L: Lepromatous	T: Tuberculoid
1	C32	P18	P19
2	C33	P21	P23
3	C36	P24	P25
4	C37	P30	P27
5	C38	P31	P29
6	C42	P35	P34
7	C43	P36	XC39
8	C44	P37	P41
9	C45	P39	P42
10	C41	P46	P49


C 1 2 3 4 5 6 7 8 9 10 NP
L 1 2 3 4 5 6 7 8 9 10 T 1 2 3 4 5 6 7 8 9 10

8.3 RESULTS

8.3.1 ATPase RT-PCR

ATPase PCR products were detected in all samples, with the exception of those from the ileum of sheep C41 and C43 of the control group, sheep P34 and P49 of the tuberculoid group, and P35 of the lepromatous group. This confirmed that mRNA had been successfully isolated and reverse transcribed from twenty five of the thirty ileal samples (figure 8.1). Streaking was observed in the sample lane for sheep P35 and may indicate contamination or breakdown of PCR products. Sample C41 was excluded from subsequent assays. Densities of the bands appeared to be slightly higher in the samples of the infected groups than in those of the control animals.

8.3.2 IFNγ RT-PCR

The autoradiograph of the probed blot of the IFNγ PCR products exhibited positive bands in the lanes of all control samples with two exceptions (C32 and C37). Positive bands were visible in the remaining seven samples from the control group. One animal of the lepromatous group (P46) and one of the tuberculoid group (P42) were negative. Otherwise, all infected animals appeared to be positive for IFNγ mRNA (figure 8.2). There was no clear-cut difference in intensity between the two groups of infected sheep (P=1), although three animals of the tuberculoid group appeared to have very strong bands (P23, P25 and P27) on the autoradiograph, and one (P21) of the lepromatous group had a weak band only. Both groups of samples from infected animals appeared to have more highly dense bands than those of the control group, and on statistical examination this was confirmed for the lepromatous

Figure 8.2 (opposite): Photograph of autoradiograph of Southern blotted IFNγ PCR products. Samples from control animals (C) are above, and those from animals of the lepromatous (L) and tuberculoid (T) groups are below, and sample-lane numbers correspond to individual animals as indicated below. N: negative-, and P: positive-control samples.
Positive bands were absent from samples C32 and C37 of the control group, P46 of the lepromatous group, and P42 of the tuberculoid group (lanes C1, C4, L10 and T8 respectively). Overall, samples from infected animals appeared to be of greater intensity than those of the control group, and this was particularly evident in three samples of the tuberculoid group (lanes T1, T2, T3).

Lane	C: Control	L: Lepromatous	T: Tuberculoid
1	C32	P18	P19
2	C33	P21	P23
3	C36	P24	P25
4	C37	P30	P27
5	C38	P31	P29
6	C42	P35	XC39
7	C43	P36	P41
8	C44	P37	P42
9	C45	P39	P49
10		P46	

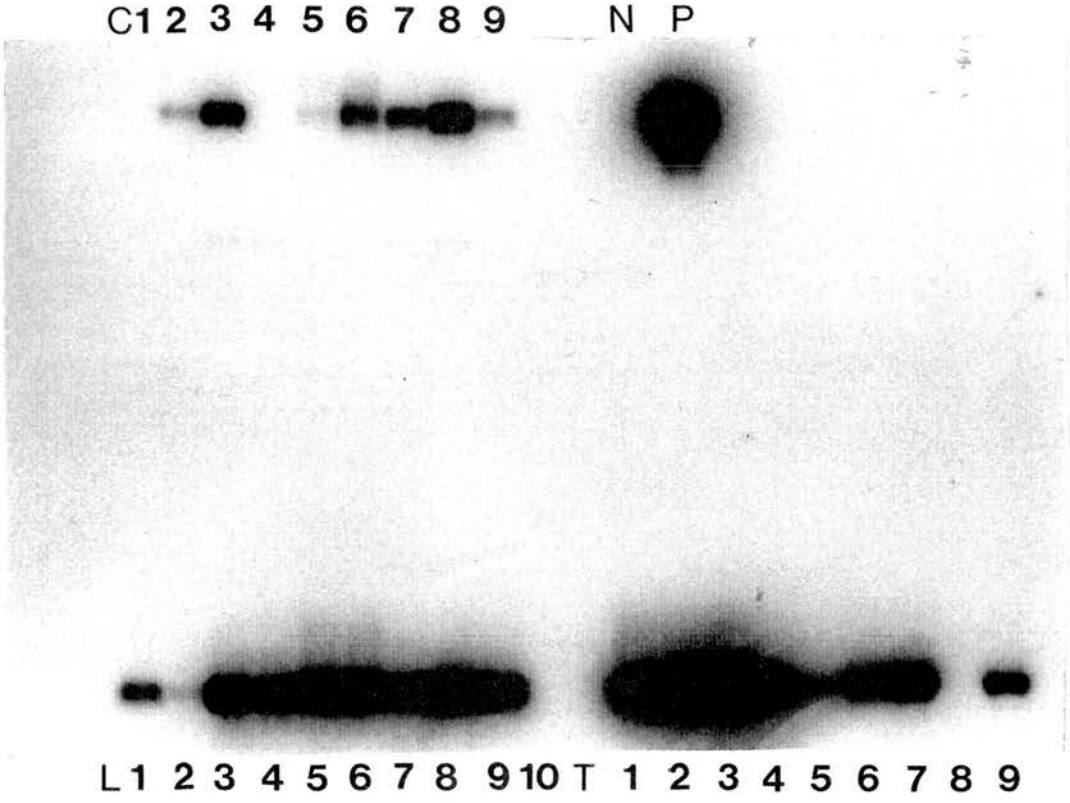

C1 2 3 4 5 6 7 8 9 N P
L 1 2 3 4 5 6 7 8 9 10 T 1 2 3 4 5 6 7 8 9

Figure 8.3: Median values assigned on assessment of mRNA levels on the autoradiographs of PCR product, and normalised to ATPase. Significant differences from the control group are denoted * ($P<0.05$) and ** ($P<0.01$), and difference between infected groups denoted # ($P<0.05$).

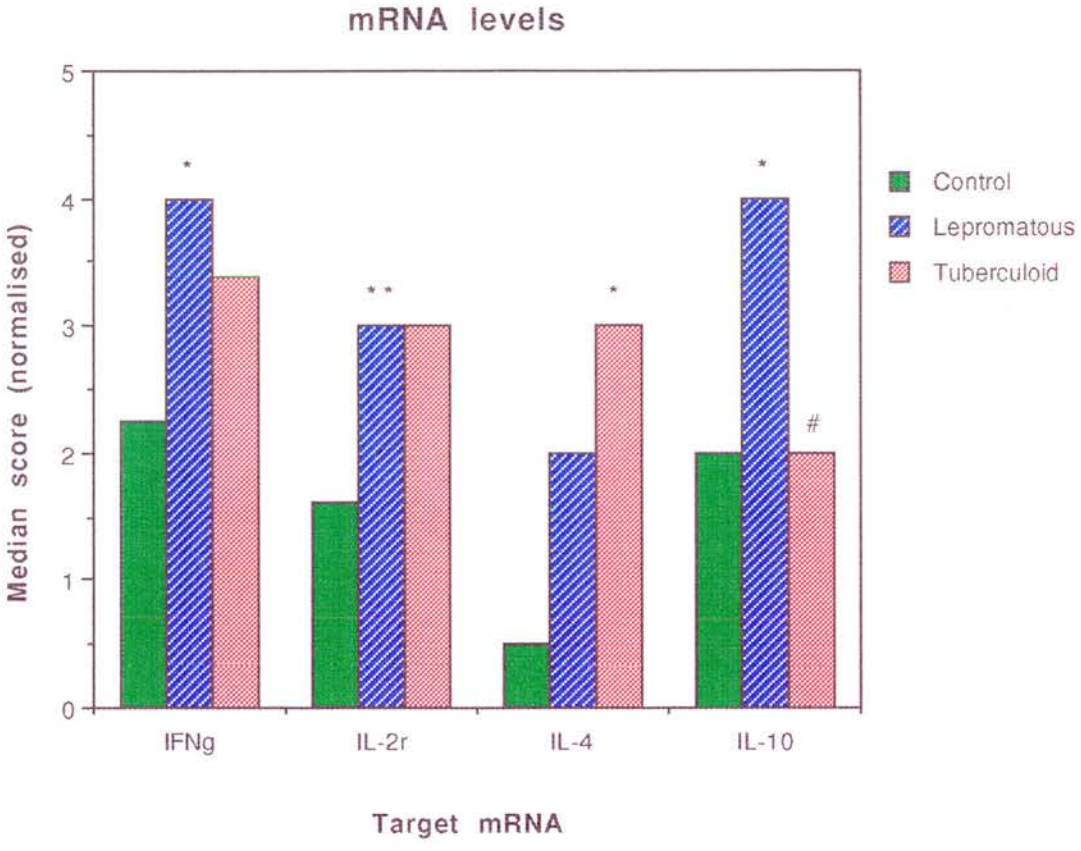
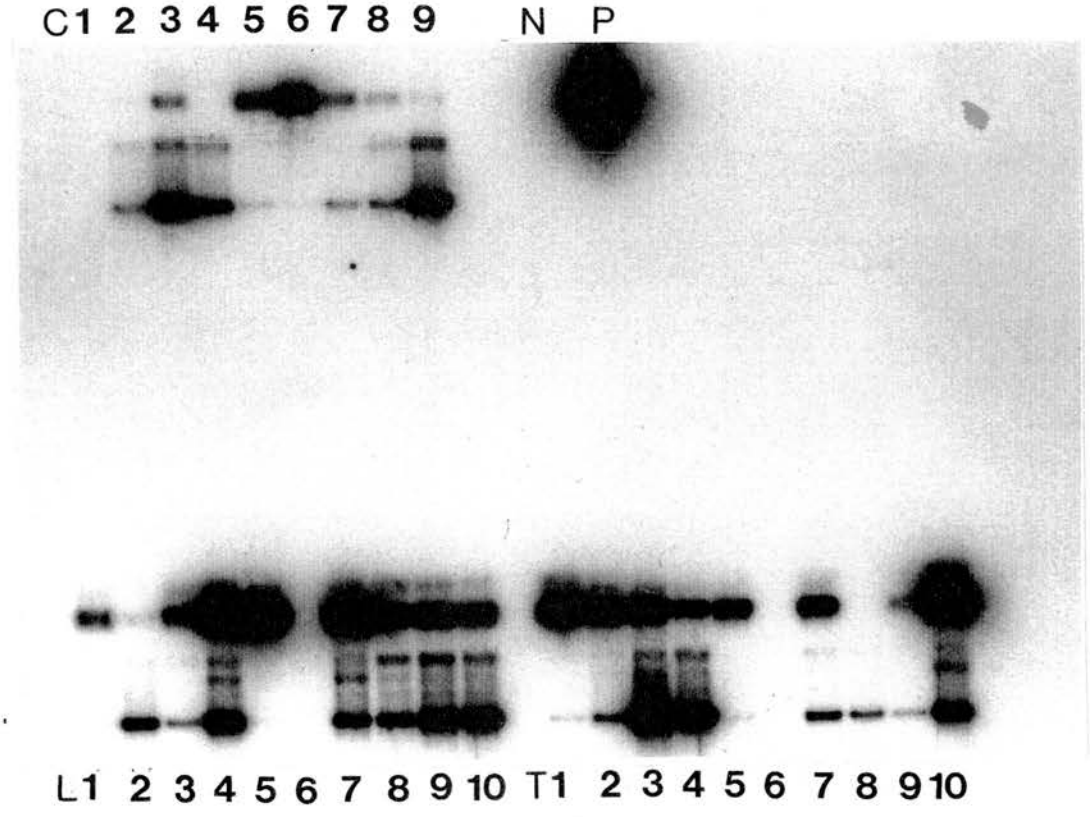


Figure 8.4 (opposite): Photograph of autoradiograph of Southern blotted IL-2r PCR products. Samples from control animals (C) are above, and those from animals of the lepromatous (L) and tuberculoid (T) groups are below, and sample-lane numbers correspond to individual animals as indicated below. N: negative-, and P: positive-control samples.
Positive bands were absent from samples C32, C33 and C37 of the control group, P39 of the lepromatous group, and P34 and P41 of the tuberculoid group (lanes C1, C2, C4, L6, T6 and T8 respectively). Bands seen in the infected groups of animals appeared overall to be stronger than those of the control group.

Lane	C: Control	L: Lepromatous	T: Tuberculoid
1	C32	P18	P19
2	C33	P21	P23
3	C36	P24	P25
4	C37	P30	P27
5	C38	P31	P29
6	C42	P39	P34
7	C43	P35	XC39
8	C44	P36	P41
9	C45	P37	P42
10		P46	P49


C1 2 3 4 5 6 7 8 9
N P
L1 2 3 4 5 6 7 8 9 10 T1 2 3 4 5 6 7 8 9 10

group ($P<0.05$), but not for the tuberculoid group ($P=0.09$). Median scores of mRNA levels for each group of animals are represented graphically in figure 8.3.

8.3.3 IL-2r RT-PCR

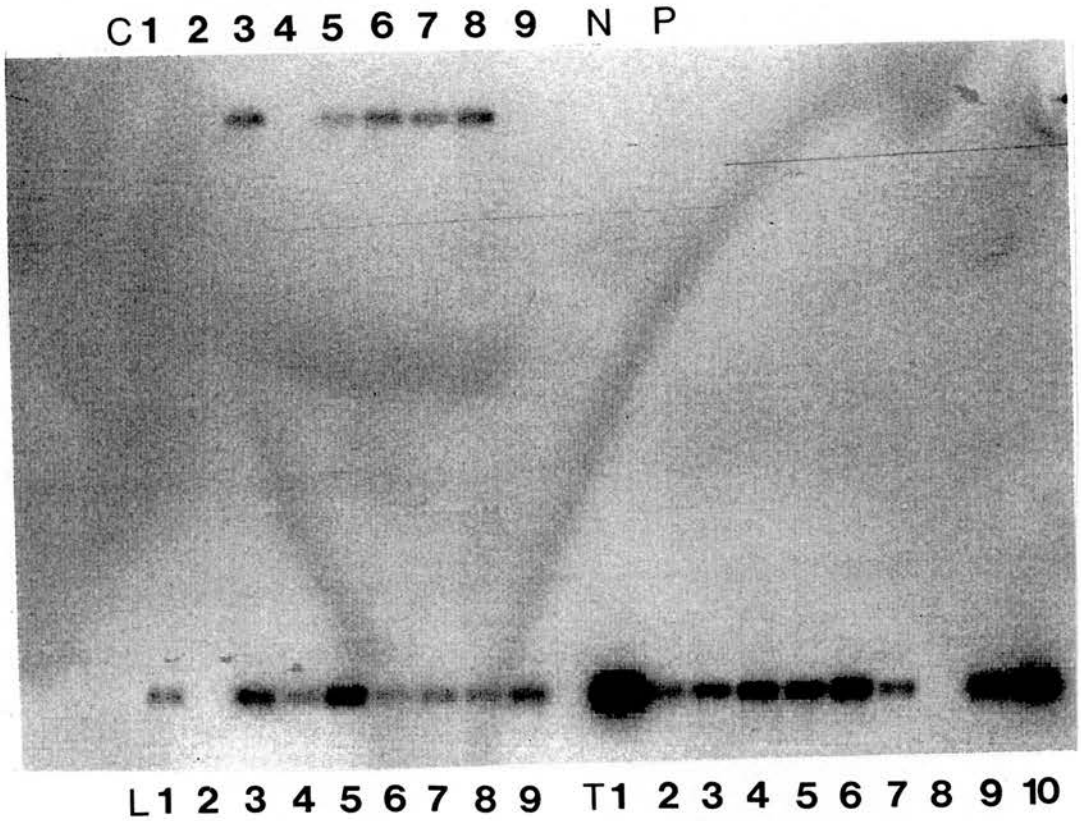
The autoradiograph of the probed blot of the IL-2r PCR products exhibited strong bands in the positive control lane. IL-2r was not detected in the sample lanes of three of the nine samples from the control group (C32, C33 and C37), and weak bands only were detectable in two samples (C44 and C45). Likewise, one (P39) of the ten samples of the lepromatous group was negative and one (P21) was very weak. Two samples of ten of the tuberculoid group (P34 and P41) were also negative (figure 8.4). Overall, there was no apparent difference in intensity of the samples between the two infected groups of sheep ($P=0.25$), although two animals of the tuberculoid group (P19 and P49) and three of the lepromatous group (P30, P31 and P35) appeared to have very strong bands. On statistical comparison with the control group, bands of the samples of the lepromatous group were found to be significantly stronger ($P<0.01$), but those of the tuberculoid group were not ($P=0.055$).

8.3.4 IL-4 RT-PCR

No band was visible in the positive control lane on the autoradiograph of the probed blot of the IL-4 PCR products, whereas this was clearly visible on the photographed gel. It is likely that the positive control used was for another set of IL-4 primers (G5287 and G5289), and this would explain the absence of this band on the autoradiograph. Five of the nine samples from control animals were positive, and the remaining four negative (C32, C33, C37 and C45) (figure 8.5). Sample P39 of the

Figure 8.5 (opposite): Photograph of autoradiograph of Southern blotted IL-4 PCR products. Samples from control animals (C) are above, and those from animals of the lepromatous (L) and tuberculoid (T) groups are below, and sample-lane numbers correspond to individual animals as indicated below. N: negative-, and P: positive-control samples. The positive control band was visible on the gel, but failed to appear on the autoradiograph, due to use of an inappropriate positive control sample which was the product of an other pair of IL-10 primers.
Positive bands were absent from samples C32, C33, C37, and C45 of the control group, P21 of the lepromatous group, and P41 of the tuberculoid group (lanes C1, C2, C4, C9, L2, and T8 respectively). Overall, the strongest bands were observed in the samples of the tuberculoid group.

Lane	C: Control	L: Lepromatous	T: Tuberculoid
1	C32	P18	P19
2	C33	P21	P23
3	C36	P24	P25
4	C37	P30	P27
5	C38	P31	P29
6	C42	P35	P34
7	C43	P36	XC39
8	C44	P37	P41
9	C45	P46	P42
10			P49


C1 2 3 4 5 6 7 8 9 N P
L1 2 3 4 5 6 7 8 9 T1 2 3 4 5 6 7 8 9 10

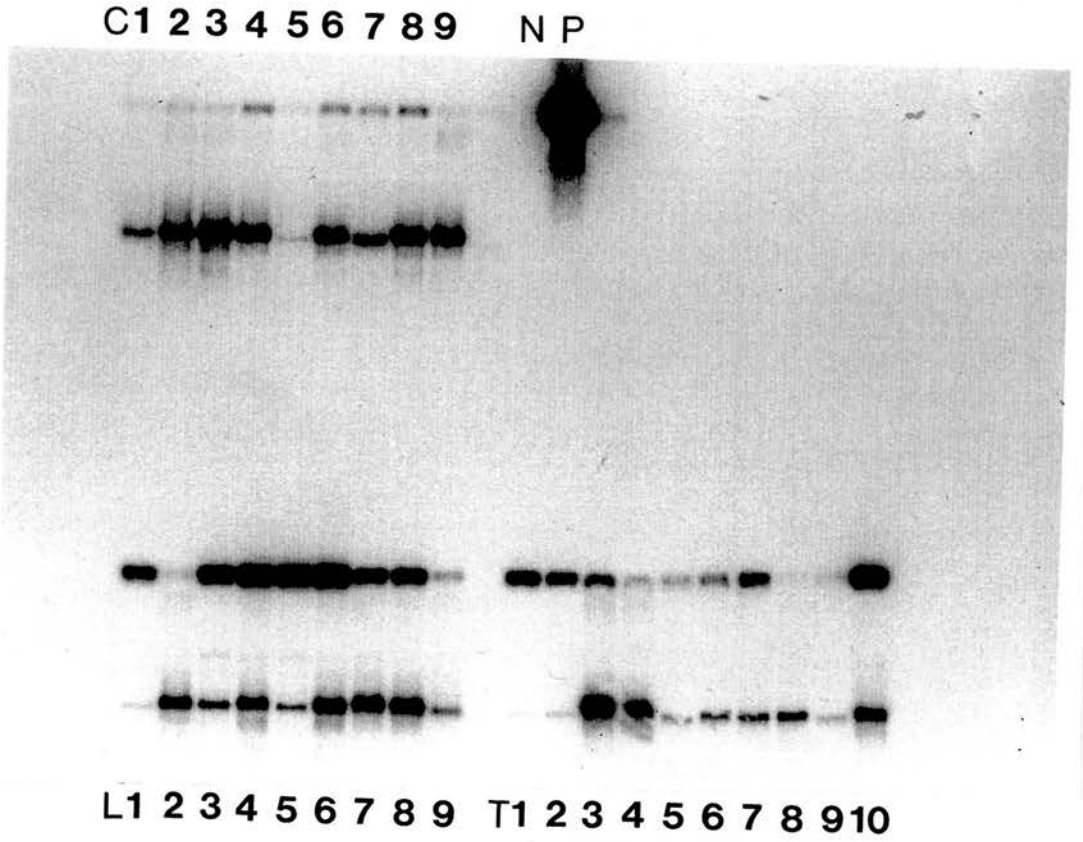
lepromatous group was not subjected to PCR for IL-4 due to insufficient cDNA sample. One sample (P21) of the lepromatous group and one (P41) of the tuberculoid group were found to be negative. Signals appeared overall to be stronger for the samples of the tuberculoid group than for those of the lepromatous group, although there was no statistically significant difference ($P=0.09$). Likewise, no significant difference was found between the scores of the control and lepromatous groups, although tuberculoid scores were significantly higher than those of the control group ($P<0.05$).

8.3.5 IL-10 RT-PCR

The autoradiograph of the probed blot of the IL-10 PCR products exhibited strong bands in the positive control lane (figure 8.6). In the sample lanes, weakly positive signals were detected in samples from the control group, with the exception of one sample (C41). Positive signals were visible all nine samples of the lepromatous group (again P39 was not included due to insufficient cDNA sample), of which four appeared very strong (P24 , P30 , P31 and P35). Two had weak signals (P21 and P46). Of the tuberculoid group, five samples had strong signals, and the remaining five had weak signals. Strongest signals were apparent in the samples of the lepromatous group, followed by the tuberculoid group, with the control group having weak signals. The scores of the lepromatous group were significantly higher than those of both control ($P<0.05$) and tuberculoid ($P<0.05$) groups. No statistically significant difference was found between the control and tuberculoid groups.

Figure 8.6 (opposite): Photograph of autoradiograph of Southern blotted IL-10 PCR products. Samples from control animals (C) are above, and those from animals of the lepromatous (L) and tuberculoid (T) groups are below, and sample-lane numbers correspond to individual animals as indicated below. N: negative-, and P: positive-control samples.
One sample of the control group was negative only (C41, lane C5). Overall, the strongest bands were in evidence in the samples of the lepromatous group.

Lane	C: Control	L: Lepromatous	T: Tuberculoid
1	C33	P18	P19
2	C36	P21	P23
3	C37	P24	P25
4	C38	P30	P27
5	C41	P31	P29
6	C42	P35	P34
7	C43	P36	XC39
8	C44	P37	P41
9	C45	P46	P42
10			P49


C1 2 3 4 5 6 7 8 9 N P
L1 2 3 4 5 6 7 8 9 T1 2 3 4 5 6 7 8 9 10

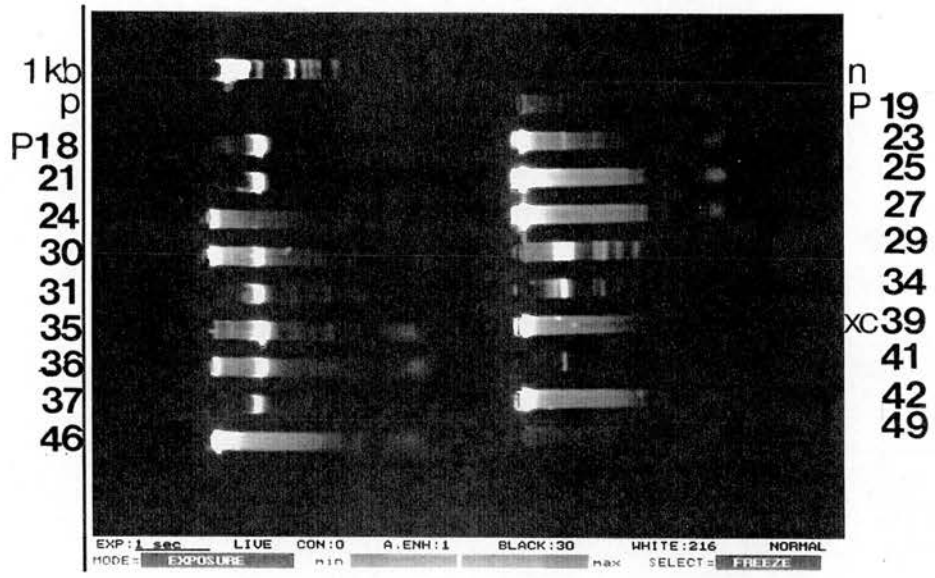
8.3.6 IS900 PCR

PCR products of 1200 bp size (figure 8.7) were visible on the ethidium bromide-stained agarose gel for samples subjected to PCR using the IS900 primers. Seven sheep of the lepromatous group (P18, P21 P30, P31, P35, P36 and P37), and five of the tuberculoid group (P19, P23, P29, P34 and P41) showed positive bands of 1200 bp size. Two of the sheep of the tuberculoid group (P25 and P42) had positive PCR results using *Mas*-specific primers for the IS901 sequence, when mycobacterial cultures were examined by co-workers at the Moredun Institute. Similarly, the remaining three sheep of the lepromatous group (P24, P39 and P46) which were negative for IS900 in this study of tissue DNA, were positive for IS900 on mycobacterial cultures. The remaining three sheep of the tuberculoid group showed immunological and serological evidence of infection, with positive *Map*-specific serum antibody and CMI responses.

8.4 DISCUSSION

IFNγ, IL-2, IL-4 and IL-10 are lymphokines, primarily produced by lymphocytes, with IL-10 in addition being produced by monocytes and macrophages. The examination of tissue levels of mRNA for these cytokines gives an indication of their production in the lesions of paratuberculosis. Both infected groups appeared, in this preliminary examination, to be associated with bands of higher density for IFNγ and IL-2r than the control group. Three samples from the animals of the tuberculoid group appeared to have very strong bands for IFNγ in particular. In addition, however, the intensity of the bands for IL-4 appeared overall to be stronger in the samples from the tuberculoid group. This may have been related to the higher densities of lymphocytes in the ileum of tuberculoid cases, and is an example of how normalisation of signals to lymphocyte-specific mRNA is likely to be important. IL-10

Figure 8.7 (opposite): Photograph of an ethidium bromide-stained agarose minigel showing PCR products of the IS900 primers. DNA samples were extracted from animals with clinical paratuberculosis. Positive PCR-product bands corresponding to 1200 bp size are visible in samples P18, P21, P30, P31, P35, P36, and P37 of the lepromatous group, and in samples P19, P23, P29, P34 and P41 of the tuberculoid group.


1kb
p
P18
21
24
30
31
35
36
37
46
n
P 19
23
25
27
29
34
xc39
41
42
49
EXP:1 sec LIVE CON:0 A.ENH:1 BLACK:30 WHITE:216 NORMAL
MODE: EXPOSURE min max SELECT: FREEZE

bands appeared to be more intense in samples of the lepromatous group, but again lymphocyte mRNA levels cannot be inferred without normalisation to lymphocyte-specific mRNA, as this cytokine is likely to be produced by macrophages, of which high densities were apparent in the ilea of lepromatous cases.

In order to better assess production of these cytokines by lymphocytes, *in-situ* hybridisation (ISH) techniques would have been preferable in that they would have permitted an assessment of mRNA production by individual cells, and of cellular distribution in relation to granulomas and to the various microanatomical compartments of the intestine. Initial attempts at ISH techniques proved to be unsuccessful, and RT-PCR was chosen as a more straightforward protocol. Furthermore, in the assessment of lymphocyte production of these cytokines, the inclusion of a lymphocyte-specific product in the RT-PCR reaction would have permitted more readily quantifiable results. For example, Yamamura *et al.* (1991) included CD3δ in their study of leprosy skin biopsies, thereby increasing the ability to normalise mRNA levels to a lymphocyte-specific mRNA, and so further discriminate lymphokines of T cell origin. However, even after normalisation of autoradiograph samples this technique would at best be semi-quantitative. Recently, competitive PCR has been described (Chelly *et al.,* 1990), where a known quantity of competitor DNA template is introduced into the PCR reaction, permitting a better and more accurate quantification of the levels of PCR product at the end of the reaction.

Nonetheless, the nature of this study was preliminary and was undertaken in order to assess the ability to examine mRNA levels in the ileal lesions of sheep with paratuberculosis. The ileal mucosa is a highly cellular tissue, with relatively high densities of lymphocytes. Therefore extraction of mRNA and reverse transcription to cDNA for PCR examination was relatively straightforward. The procedure was complicated only by the high levels of mucus in the tissue samples, and the relative

paucity of specific ovine reagents. Differences in the levels of mRNAs, however, may be related to the densities of cells present in the ilea of the three groups of animals, and of the relative densities of different cell types. Indeed, lymphocyte densities were found to be significantly higher in the ilea of sheep with the tuberculoid form of the disease (chapter 3).

Yamamura *et al.,* 1991 found that resistance and susceptibility, as defined by paucibacillary and multibacillary forms of leprosy, correlated with distinct patterns of cytokine mRNA levels. Monokine mRNAs were more abundant in tuberculoid than in lepromatous lesions, and type-1 lymphokine mRNAs (IFNγ, IL-2 and TNFβ) were highly expressed in tuberculoid lesions, but scarce in lepromatous lesions. In contrast, levels of IL-4, IL-5 and IL-10 were higher in lepromatous lesions. Furthermore, in an examination of the immune reactions of leprosy, Yamamura *et al.* (1992) noted that in reversal reactions, which are characterised by influx of $CD4^+$ cells and increased CMI and bacillary clearance, mRNA for IFNγ, IL-2 and TNFα were progressively dominant, with concomitant decreases in mRNA for the type 2 cytokines IL-4, IL-5 and IL-10. In contrast, ENL reactions were characterised by dominance of IL-4 and IL-5, and progressive increases in IL-6, IL-8 and IL-10, which is in keeping with the underlying immediate-type hypersensitivity reaction in this phenomenon.

IL-10 was found to be prominent in the lesions of lepromatous leprosy, but produced in relatively low levels by T-cell clones isolated from lesions (Sieling *et al.,* 1993). It was suggested in that study that *M. leprae* induces IL-10 production, primarily by macrophages, and that IL-10 inhibited *M. leprae*-specific T-cell proliferation and release of IFNγ, TNFα and GM-CSF.

No such clear cut differences were observed in this study. However, the gut, unlike skin, is a mucosal organ with high densities of immune cells present in normal

tissue, and with underlying functions of immune surveillance and antibody production at a mucosal surface. Cells of the gut immune system may be expected to be of distinct functional type from those of the skin immune system, and it has been postulated that cells of Th0 phenotype may be dominant in normal gut (Romagnani, 1994), and that Th2 cell clones are responsible for the promotion of antibody production at this site. James *et al.* (1990), found high levels of mRNA for IFNγ, IL-2, IL-4 and IL-5 in isolated intestinal LPL (from non-human primates) which had been activated *in vitro* with ionomycin and PMA (phorbol myristute acetate), and concluded that LPL had high capacity to express these cytokines in comparison with cells isolated from peripheral blood, peripheral lymph node and spleen. In the same study, it was also observed that IL-2 induced proliferation of isolated LPL. Regulation of the local immune responses is likely to be of utmost importance in the gut immune system, due to the necessity to either respond to, or be tolerant to, a wide range of pathogens and antigens in the GI tract, and it would be expected that cytokines would be important effectors of these mechanisms. It has been found that mucosal biopsies from humans affected by Crohn's disease showed increased levels of mRNA for IFNγ, IL-2 and TNFα, whereas those from patients with ulcerative colitis had increased cytokine transcripts for IL-4 and IL-10 (Mullin *et al.,* 1993).

It would be worthwhile to examine a wider array of cytokine mRNA in the lesions of paratuberculosis, in particular levels of IL-12 which has a putative role as promoter of type 1 responses in other species. IL-12 was first identified by Kobayashi *et al.* (1989) as a natural killer cell stimulatory factor, and has since been found to be instrumental in the differentiation of T cells, initiation of CMI, and the generation of Th1 cells through induction of IFNγ and inhibition of IL-4, and proliferation of Th1 cells (reviewed by Trinchieri and Scott, 1995). The activity of IL-12 is subject to downregulation by IL-10 (D'Andrea *et al.,* 1993), and IL-10 has been shown to downregulate Th1 responses and expression of costimulatory molecules in

Mycobacterium tuberculosis infection, and also the expression of MHCII by macrophages (Gong *et al.,* 1996; Bogdan *et al.,* 1991). The role of IL-12 in mycobacterial disease was reviewed by Modlin and Barnes, (1995), who described an RT-PCR study of leprosy lesions, in which up to 50-fold higher levels of IL-12 mRNA were detected in tuberculoid compared with lepromatous lesions, with a 10-fold higher percentage of IL-12-positive cells in tuberculoid than in lepromatous granulomas. They proposed that in tuberculoid patients IL-12 production by macrophages augments a type 1 response, with $CD4^+$ cells producing IFNγ resulting in macrophage activation. Lepromatous lesions, however, are characterised by infiltration of $CD8^+$ cells which produce IL-4, and macrophages which produce IL-10, with resultant inhibition of IL-12 production and suppression of CMI. TNFα, IL-1β and IL-6 mRNA levels were examined and found to be increased in the intestinal tissues of sheep with lepromatous lesions of paratuberculosis, compared with normal control sheep (Alzuherri *et al.,* 1996), suggesting that these pro-inflammatory cytokines may contribute to the intestinal pathology in these animals. Animals with tuberculoid lesions, however, showed elevated levels of mRNA for TNFα only. Parida and Grau, (1993), reviewed the actions of TNFα in mycobacterial disease, and described how TNFα, in conjunction with IFNγ, has been shown to result in restriction of intracellular mycobacterial growth, and to be instrumental in formation and regulation of granulomas in mycobacterial diseases. However, TNFα is also contributory to tissue damage and cachexia.

It is apparent that cytokine control in mycobacterial infections is relatively complex, and that ileal lesions of paratuberculosis in particular may involve interactions of different cytokines that are far from straightforward, and that these would certainly merit further study.

CHAPTER NINE

PARATUBERCULOSIS IN A HERD OF GOATS

9.1 INTRODUCTION

Paratuberculosis in the goat has been described by a number of authors (Baas, 1977; Collins *et al.,* 1984; Fodstad and Gunnarsson, 1979; Lenghaus *et al.,* 1977; Morin, 1982; Saxegaard, 1990). Paratuberculosis may be a relatively common condition of goats, as a survey of 2635 goats in Portugal suggested that 10% had serological evidence of infection (Amado *et al.,* 1994). The clinical diagnosis of paratuberculosis in goats is problematic, with early infections often being asymptomatic, and with the later stages associated with only non-specific signs such as ill-thrift and anaemia. In contrast with sheep, goats are often kept for milk production and decreasing milk yields have been recorded. As in sheep, faeces are usually normal in character except in the very terminal stages of the disease during which soft faeces, and only very infrequently diarrhoea, have been noted. Animals have been noted to be very weak and moribund in the terminal stages of infection. Medical findings have included hypoproteinaemia, non-regenerative anaemia and depressed serum calcium and magnesium levels (Morin, 1982). On necropsy examination, gross lesions of paratuberculosis can be less immediately apparent than in cattle and sheep. Lymphadenomegaly of MLN, occasionally associated with oedema has been the most frequently described gross change, and lesions of caseous necrosis and mineralisation have been observed in these nodes. One report described peritonitis with deposition of fibrinous tissue (Lenghaus *et al.,* 1977). Gross changes in the intestinal tract have been noted, but appear to be confined to mild thickening of the gut wall, and cording of the lymphatic vessels. The common findings in ovine paratuberculosis of marked thickening of the intestinal wall and corrugation and

crevicing of the mucosal surface, have been less frequently noted in goats. Histopathological findings have been described for areas of the small and large intestine, MLN and liver, and have included a range lesions with different degrees of severity, varying from mild lymphoid cell and macrophage infiltrates to extensive macrophage infiltration of the lamina propria and loss of normal intestinal architecture.

The immune responses of caprine hosts to *Map* infection do not appear to have been examined, with the exception of one study of the humoral and LSA responses to vaccination (Molina *et al.,* 1996) in which positive lymphoproliferative responses to *Map* antigen were demonstrated after vaccination. Another study evaluated immune responses in a small group of goats and observed stronger reactions to intradermal testing in subclinical or "reactive" cases, but progressive antibody production and lowered intradermal responses in clinical, or "unreactive" cases (Paliwal *et al.*, 1985).

A herd of goats with endemic paratuberculosis was acquired by the Moredun Research Institute. The aim of this study was the evaluate the gross and histological lesions in these goats, and to assess the CMI responses of PBL by LSA, and serum antibody levels by ELISA. In addition, lymphocyte isolation from tissues was performed in order to provide an indication of the cellular populations in goats with paratuberculosis.

9.2 MATERIALS AND METHODS

9.2.1 Animals

All goats came from the same herd, which was acquired and subsequently kept at the Moredun Research Institute, and consisted of 30 goats of the Nubian breed. All of the animals used in the pathological survey were female and adult.

9.2.2 Gross and histological pathology

Tissues were collected fresh at necropsy examination as described in section 2.2.2, and processed routinely as described (sections 2.2.3-4) for HE and ZN staining. Sections were examined and were scored using the system described (section 2.2.5) by the same pathologist as before (Dr C.J. Clarke).

9.2.3 Lymphocyte isolation and FACS analysis

LPL, IEL, MLNL and PBL were isolated and purified according to the procedures described in sections 4.2.5-8. The same panel of MAbs was used as for the study of sheep lymphocytes (table 4.1). Cross-reactivity between ovine and caprine species has been previously established for a number of MAbs (Naessens *et al.,* 1993; Hein *et al.,* 1991), and others were used here and compared with the data available for sheep. Fluorescent staining and FACScan analysis of cells was performed as previously described (sections 4.2.9-10).

9.2.4 Serum antibody ELISA and LSA

Serum *Map*-specific antibody levels were evaluated by means of the *M. phlei*-absorbed ELISA, according to the procedure described in section 5.2.3. LSA was performed as described in section 5.2.4.

9.3 RESULTS

9.3.1 Pathological findings

Gross and histological changes are shown in table 9.1. Seventeen goats were submitted for necropsy examination. A total of four goats had visible gross lesions associated with *Map* infection, either of gut, MLN or lymphatics. Gross lesions of the gut were visible only in two of the goats subjected to necropsy examination. Slight thickening of the intestinal wall was evident in two goats only, one of which had thickened ileum, and the other had thickening of the jejunum and not of the ileum. None of the goats examined had pigmentation of the mucosal surface. None of the characteristic features of ovine paratuberculosis, such as ridging and crevicing or segmental thickening, were observed, nor was oedema a feature of the gross intestinal changes. Two animals showed prominence and cording of the serosal lymphatics.

Three goats had gross changes of the MLN, which was evident as severe node enlargement in two of the goats, and moderate enlargement in the third. Two of the animals with gross enlargement of the MLN had calcified and caseous, necrotic areas visible within the opened node.

Table 9.1: Summary of immunological and pathological findings for goats with evidence of *Map* infection.

Goat	*LSA*	*Antibody*	*Gross Pathology*			*Histopathology*							
	SI	EAU	Gut	MLN	Lymph	LIM	EpIM	EpDist	AF%	AF No.	MLN	MLN AF No.	Liver
A052	0.42	431	0	3	0	1	2	D	2	2	3	2	1
A053	0.93	271	1	3	1	2	1	F	2	2	1	1	2
A063	3.52	55	0	0	1	1	0	-	0	0	0	0	0
A080	6.61	33	0	0	0	1	1	F	0	0	0	0	0
A066	70.02	51	0	0	0	2	1	F	0	0	3	0	0
A060	2.6	122	0	0	0	2	1	F	2	2	2	0	1
A081	12.21	39	1	2	0	1	1	F	1	1	3	3	1

Lymph: lymphatic changes; LIM: lymphocytes in mucosa; EpIM; epithelioid macrophages in mucosa; EpDist: distribution of epithelioid macrophages in mucosa; AF%: percentage of macrophages infected with AFB; AF No: number of AFB in macrophages; MLN: histopathology in MLN; MLN AF No: number of AFB in macrophages of MLN lesions; Liver: histological lesions in liver.

Seven animals had histological evidence of change associated with *Map* infection. All seven animals had a mild (4 animals) to moderate (3 animals) lymphoid-cell infiltrate in the mucosa of the ileum or jejunum. One animal had histological lesions present in the jejunum only, and a further animal had lesions in the jejunum and ileum. The remaining five animals had lesions of the ileum only. A mild infiltrate of epithelioid-type macrophages was seen in the mucosa in five goats, a moderate infiltrate was evident in one further animal, and one goat had no evidence of an infiltrate of these cells present. Of the six animals with epithelioid cells present, one animal had diffuse distribution of these cells, and the remaining five showed a focal pattern of macrophage aggregates. Three of the seven animals with histological lesions had moderate numbers of AFB in the epithelioid macrophages, with corresponding moderate numbers of these cells being infected by AFB. Lesions were found in the submucosa in two goats, and in the serosa in one goat only, and consisted of mild to moderate numbers of lymphocytes in the submucosa with focal aggregates of epithelioid cells seen in one animal, and lymphoid cell infiltrate into the serosa.

Five of the seven animals with intestinal histopathology also had histological lesions of the MLN. Three of these animals had severe granulomatous change, including two animals with caseous necrosis and evidence of calcification. One of these animals had free AFB visible within the caseous necrotic centres of the MLN, and a further animal had Langhans-type giant cells present. One goat had moderate and another mild focal granulomatous change present in the MLN. Of the five goats with lesions in the MLN, three had evidence of AFB present in the granulomas. Four animals also had granulomatous change present in the liver, and all four of these goats had granulomas in the MLN. Only one animal of those with granulomatous change in the liver had a few AFB visible in this organ (goat A052).

9.3.2 FACS analysis of PBL isolated from goats with and without lesions

The PBL isolated from goats with histological lesions were compared with those from goats with no apparent lesions of paratuberculosis (table 9.2). The median percentage of $CD4^+$ cells was found to be lower in the animals with lesions than in those without ($P<0.05$). Interestingly, the group of goats with lesions was associated with higher percentages of B cells ($P=0.01$), in keeping with the similar observations made in sheep with paratuberculosis. The percentages of cells staining positive for MHCII, both DQ (VPM36, $P<0.01$) and DR (VPM54, $P<0.05$) haplotypes, was found to be higher in the group of animals with lesions, and this is consistent with the higher percentages of B cells in this group. The same pattern was observed for the pan-MHCII MAb (SW73,2), but the difference was outside the range of statistical significance at $P=0.06$.

9.3.3 FACS analysis of LPL, IEL and MLNL isolated from goats with lesions

The percentages of LPL, IEL and MLNL are shown in table 9.3. Only animals with histological lesions of paratuberculosis were subjected to isolation and analysis by FACScan, as no healthy, normal control goats were available for comparison. On comparison with the ovine FACS data included in chapter 4, T-cell subsets appeared to be present in similar proportions to those described for sheep, with the exception of the percentage of $CD8^+$ cells in the MLNL which appeared to be higher than would have been expected from the sheep data.

Table 9.2: FACS analysis of PBL isolated from goats with histological evidence of paratuberculosis, and from goats with no histological lesions detectable after necropsy examination. Values are percentages of positively stained cells (median and range). ND: not determined.

MAb	*No lesions (n=9)*	*Lesions (n=6)*
SBUT4	16.6 (11.2,25.1)	10.1 (7.6,18.8)*
SBUT8	15.8 (9.5,30.9)	17.5 (9.6,47.9)
86D	6.2 (3.5,12.9)	5.5 (2.6,8.0)
CC15	8.7 (4.0,13.7)	6.6 (3.9,9.8)
ST1	ND	10.5 (6.1,19.1)
VPM8	46.8 (27.8,58.6)	65.2 (45.9,80.7)*
VPM19	99.0 (87.6,99.8)	99.3 (97.0,99.5)
SW73,2	58.4 (43.7,77.3)	73.8 (61.7,86.1)
VPM36	50.6 (38.4,65.3)	70.0 (55.8,77.5)**
VPM54	51.2 (41.6,64.7)	66.7 (54.0,82.5)*

Table 9.3: FACS analysis of cells isolated from ileum and MLN of goats with histological evidence of paratuberculosis. Values are percentages of positively stained cells (median and range).

MAb	*n*	*LPL*	*n*	*IEL*	*n*	*MLNL*
SBUT4	4	54.5 (34.8,63.6)	3	22.3 (22.3,28.3)	4	20.2 (11.3,24.5)
SBUT8	4	29.6 (26.8,42.2)	3	50.0 (46.3,64.0)	4	27.7 (17.4,34.3)
86D	4	6.1 (4.3,7.5)	3	8.4 (6.2,8.6)	4	2.8 (2.5,3.9)
CC15	4	5.9 (5.2,6.5)	3	6.5 (5.8,7.0)	4	2.8 (2.5,3.8)
ST1	3	60.2 (48.4,63.4)	3	22.6 (21.6,27.2)	4	19.9 (13.2,25.1)
VPM8	4	6.3 (1.8,14.3)	3	3.0 (1.5,21.2)	4	59.0 (51.5,64.8)
VPM19	3	96.9 (96.1,98.8)	3	96.7 (94.2,98.4)	4	98.0 (92.0,98.7)
SW73,2	3	82.0 (81.5,83.8)	3	85.7 (79.2,86.6)	4	81.3 (64.4,87.0)
VPM36	3	60.3 (36.4,61.8)	3	34.4(10.3,49.5)	4	71.0 (55.6,78.8)
VPM54	3	35.8 (25.7,65.2)	3	41.0 (31.0,54.5)	4	65.9 (51.8,68.5)

Table 9.4: LSA SI and EAU values determined for goats examined by necropsy with and without lesions of paratuberculosis, and animals tested but not necropsied.

Group	*Goat number*	*LSA SI*	*EAU*
Lesions of paratuberculosis	A052	0.42	431
	A053	0.93	271
	A060	2.60	122
	A063	3.52	55
	A066	70.02	51
	A080	6.61	33
	A081	12.21	39
No lesions noted	A054	9.70	53
	A056	50.23	65
	A058	9.37	69
	A059	3.22	130
	A061	14.61	288
	A067	91.90	59
	A069	4.57	62
	A073	3.29	52
	A074	60.46	23
	A078	49.21	-ve
No pathological examination	A055	117.30	39
	A057	41.19	114
	A062	4.24	47
	A064	74.71	-ve
	A065	57.97	62
	A068	59.47	39
	A070	37.61	63
	A071	21.55	67
	A072	26.63	31
	A075	56.19	36
	A076	29.98	27
	A077	168.78	72
	A079	58.81	52

9.3.4 Serum antibody and LSA levels

Serum antibody levels and LSA SI values are included in table 9.4. Correlations between data sets were examined using Spearman's Rank Correlation test. In the group of animals with histological lesions of paratuberculosis, a strong negative correlation was found between EAU and LSA SI values ($r=-0.875$, $P<0.05$). Strong negative correlations were also noted between LSA SI and AFB number, and between LSA SI and the percentage of macrophages with intracellular AFB (both $r=-0.772$, $P<0.05$). Conversely, strong positive correlations were found between EAU and AFB number, and between EAU and percentage of macrophages with intracellular AFB (both $r=0.772$, $P<0.05$). For the group consisting of goats which were subjected to necropsy examination, but with absence of histological lesions, a weak negative correlation was observed between LSA SI and EAU ($r=-0.345$, not significant). When all animals necropsied were considered as a single group, a modest negative correlation was noted ($r=-0.542$, $P<0.05$), and when the data available for the entire herd was examined, a modest negative correlation was also found ($r=-0.430$, $P<0.05$).

Goats with and without lesions at necropsy were compared, using the Mann-Whitney test, on the basis of LSA SI and EAU values, and no significant differences were found between the median EAU values, nor between the LSA SI values for each group.

9.4 DISCUSSION

Diagnosis of paratuberculosis on the basis of clinical signs is difficult in goats. Four goats of the seven in which lesions of paratuberculosis were detected were noted to be weak on clinical examination ante-mortem. One animal was very thin (condition

score of 1), but the others were in moderate condition (condition scores of 2). Soft faeces were noted in only one of the goats, which happened to be a weak goat and had the lowest condition score (goat A052). Even on necropsy examination, gross lesions proved to be few, with only four of the goats having grossly visible lesions, which were of the MLN in three animals, and in the gut of only two. Three goats had no detectable macroscopic lesions, and one further goat showed mild thickening of the serosal lymphatic vessels only. The paucity of macroscopic lesions is in keeping with the findings of Fodstad and Gunnarsson (1979) who detected gross lesions in 65% of goats in which infection was demonstrated by mucosal smears, bacteriological culture or histological examination. The most frequent finding by those workers was the presence of enlarged MLN in 35% of infected goats, with calcification or caseation noted in 21%. Similarly, in a survey of 273 goats in which infection was confirmed, 52% of animals showed no gross changes of the small intestine. Corrugation of the intestinal mucosa is frequently observed in ovine paratuberculosis and was found in 38% of diseased sheep in this study (section 2.3.4), but was not noted in any of the seven goats with lesions. Fodstad and Gunnarsson (1979) found corrugation of the mucosa in only 7% of infected animals, and all animals that demonstrated this feature proved to be infected, suggesting that in goats it may be a feature specific to paratuberculosis. In sheep, grossly visible thickening of the ileal wall was observed in 100% of multibacillary and 85% of paucibacillary cases, however this was a feature in only two of the seven goats, and was noted in 40% of infected goats by the aforementioned workers. Histologically, on the basis of AFB burden, three of the goats conformed to the multibacillary form of the disease, with the remaining four being more consistent with the paucibacillary classification. However, only one animal was observed to have diffuse distribution of macrophages, which was a characteristic of multibacillary disease in sheep. Extension of cellular infiltrate into the submucosal tissue was a common finding in sheep, but was observed in only two of the goats (A053 and A060), both of which had multibacillary lesions. The intracellular AFB

burdens observed in the goats were lower than those noted in sheep. The maximum score assigned for this parameter was 2, whereas 94% of ovine multibacillary cases had a score of 3. Lesions were observed in the MLN of five of the seven goats, and were in the form of focal granulomas in the cortical and subcapsular areas. Caseous necrosis and calcification were seen in the nodes of two animals, and this has been reported by other authors (Morin, 1982; Fodstad and Gunnarsson, 1979; Lenghaus *et al.,* 1977; Collins *et al.,* 1984). The presence of caseous necrosis and mineralisation is quite different from the manifestation of paratuberculosis infection in cattle and sheep, in which such changes have been reported very rarely. There was no evidence of caseous necrosis in any of the sheep in this study (section 2.4), and any mineralisation of MLN which was encountered could not conclusively be attributed to *Map* infection. The presence of such lesions in goats is most likely due to a difference in the response of the host-species to *Map* infection, although it is possible that differences between species-specific *Map* strains could be involved. It is interesting that only seven of the seventeen animals examined by necropsy had histological lesions, despite evidence of infection on assay of serological and cellular responses. This may suggest an exposed or resistant state in these animals, or they may simply be cases in the early stages of infection. In cases with minor and sporadic histological lesions, there may be difficulty in finding affected areas in the entire length of the intestinal tract, especially in the absence of gross lesions, which are not in evidence in the majority of caprine cases of paratuberculosis. This may result in lowered probability of detecting lesions in early or mild cases, and may be compounded by the fact that, unlike in sheep, lesions may not always be present in the ileum. All sheep were found to have lesions in the ileum (table 2.3), and this is considered to be the primary site for lesions. Vialard *et al.,* (1994), stressed the importance of taking multiple samples from a range of areas of the goat small intestine without favouring the area of the ileocaecal valve, which traditionally has been considered to be the most frequently affected area in cattle

and sheep. As in sheep, focal granulomatous change was seen in the liver of a number of the goats, and AFB were found in the liver lesions in one of these cases.

On examination by FACS of PBL isolated from goats with and without detectable lesions of paratuberculosis, the main finding was significantly increased percentages of sIg positive staining cells in the group in which lesions were observed. Similar changes were observed in ovine paratuberculosis. $CD4^+$ cells were noted to be present in lower proportions in this group, and this may be due to selective recruitment of these cells to the affected tissues, or as a result of the altered percentages of B cells.

The percentages of lymphocyte subsets in IEL (cells isolated from the initial EDTA incubation of ileal mucosa), LPL (cells isolated from the enzymatic digestion of ileal mucosa), and MLNL have been included in order to provide an indication of the proportions of cell subsets, although there is no data available for normal, healthy control animals to permit comparison. Nonetheless, the data given for these tissues appeared on preliminary analysis to be comparable with the ranges observed for ovine tissues.

It is interesting that in the herd of goats several significant negative correlations were found between the data for serological and cellular responses. It would appear that within this group of animals, either CMI responses or antibody responses are dominant, and that there is significant negative correlation between the two. The strongest negative correlation was observed in the group of animals with histological lesions, and despite the sample size being relatively small (seven goats) this proved to be statistically significant. As observed for the sheep, positive correlations were noted between the presence and number of AFB and serum antibody levels. Corresponding negative correlations were found between lymphocyte proliferative responses and AFB number.

This was a herd with endemic paratuberculosis, and infection was presumably spread within the group from older to juvenile animals. The majority of animals had a proliferative response to JPPD, with only three animals having negative LSA SI levels (SI < 3.0), and all three of these goats in fact had histological evidence of infection. The question remains whether the goats with positive LSA SI values were infected with *Map* organisms, or whether they had been exposed to infection, and had mounted a successful immune response resulting in clearance of the pathogen. The presence of cellular responses to *Map* antigen may therefore equate to either infection or to immunity. In an ideal situation, monitoring of a herd of goats, or a flock of sheep, would best be done by considering both CMI and serological testing. The presence of serum antibody has been shown correlate with the presence of AFB, and may be a reliable indicator of infection, especially using the absorbed ELISA and AGID tests which have been shown to be highly specific and highly sensitive (Clarke *et al.,* 1996), at least in ovine paratuberculosis and in cases with multibacillary forms of the disease. The use of the AGID test has been described as having good sensitivity for the diagnosis of caprine paratuberculosis when compared with histological findings (Vialard *et al.,* 1994), although there is evidence that the ELISA test may be better suited to the diagnosis of early and paucibacillary infections (Clarke *et al.,* 1996). In the same study, the specificity of both AGID and ELISA tests was considered to be absolute, with no false-positive results being recorded.

The findings of this study suggest that a number of differences exist between the manifestations of paratuberculosis in goats and sheep, on the basis of both macroscopic and histological pathology, and in the immune reactions observed. The study of an entire herd of goats showed a relative absence of clinical signs and gross lesions in the presence of a range of histological manifestations and CMI and antibody responses, and raises the question of the existence of an exposed and resistant state. This provided a different perspective from the examination of clinically affected

individual animals which were in the terminal stages of disease. Nonetheless, in similitude to ovine paratuberculosis, a number of animals was presented which had different immunological and pathological findings in direct response to infection with the same pathogen.

CHAPTER TEN

GENERAL DISCUSSION

The aims of this thesis were to evaluate the lesions of paratuberculosis in sheep and goats, and to relate these to the immune responses of the infected animals.

A range of gross and histological lesions was observed, and clinical cases of paratuberculosis were initially divided into two groups on the basis of AFB number, and were termed multibacillary and paucibacillary accordingly. Examination of the cell types in the ileal lesions led to the conclusion that the AFB burden correlated well with the cellular population present. Animals of the paucibacillary group were found to have significant lymphoid cell infiltrate, with the presence of Langhans type giant cells noted in a number of cases, whereas the multibacillary group was associated with the presence of large macrophages with heavy intracellular bacillary burdens. With reference to the description of the polar forms of leprosy, and to the observations of other workers in paratuberculosis, these groups were designated tuberculoid and lepromatous cases respectively. The clinical presentation of ovine cases differed little between the two infected groups, as did grossly visible lesions, with the exception of the presence of pigment, and of a greater degree of ileal-wall thickening in many of the multibacillary cases. This classification of ovine cases of paratuberculosis provided the basis for examination of the immune responses in both groups of infected animals.

Once the presence of two distinctive groups of cases was established, it followed that the T-lymphocyte subsets in the ileal lesions should be evaluated, and this was attempted using immunoperoxidase staining, which permitted enumeration of the respective subsets. It was confirmed that tuberculoid cases were associated with a

significant infiltrate of T cells, with an increased proportion of $CD4^+$ cells, the helper-cell phenotype, and a corresponding increase in the CD4:CD8 ratio. This finding was in keeping with observations on the cellular infiltrate of tuberculoid leprosy. Lepromatous cases, on the other hand, showed little difference in the proportions of $CD4^+$ and $CD8^+$ cells, but an increase in the relative percentage of $\gamma\delta$ TCR^+ lymphocytes, and suggested that this subset may be important in the pathogenesis of this form of the disease. The densities of the $CD4^+$ and $CD8^+$ subsets were found to be lowered in the ileum of this group, probably as a result of the space-occupying effect of a predominantly macrophage infiltrate. There exists no previous report on the phenotype of cells present in the lesions of paratuberculosis.

Cell isolation from the ileum, MLN and blood was undertaken in order to permit further phenotypic and functional analysis of the cellular populations of these tissues, and a modified protocol was developed for the isolation of ileal cells which resulted in adequate cellular yields of good viability. Analysis of LPL by flow cytometry confirmed the observations made on immunohistochemical staining that tuberculoid cases appeared to be associated with higher percentages of $CD4^+$ cells, whereas $T19^+$ cells were present in higher percentages in the lepromatous group. No differences were apparent in the LP proportions of B cells, based on sIg and MHCII staining. On analysis of lymphocyte phenotype of MLN, which represented a secondary site of infection, no significant differences were noted in the cellular populations, with the notable exception of an increase in the percentage of $\gamma\delta$ T cells in the MLN of lepromatous cases, in keeping with the corresponding elevation in the proportion of this subset in the LP of these cases as detected by immunoperoxidase staining. The most notable finding on phenotypic analysis of PBL was a dramatic elevation in the percentage of sIg^+ B cells in the circulation of both groups of infected animals, and this was reflected in the increased percentages of MHCII-positive cells. Furthermore, a study of serum antibody levels by ELISA confirmed that both groups of diseased

animals had high levels of circulating antibody. On examination by AGID test, fewer animals with tuberculoid lesions had antibody detectable by this method, and a strong correlation was noted between AFB number and the presence of serum antibody.

The presence of differences in the lesions and cellular population of the ileum in each group of animals prompted study of the animals' cell-mediated immunity, and this was evaluated by examination of lymphoproliferation, and production of IFNγ and IL-2, in response to stimulation with JPPD. Highest proliferative responses were observed in the tuberculoid group, and in contrast, cells isolated from the lepromatous group showed very poor proliferation to mycobacterial antigen, with no significant difference noted between this and the control group. Similarly, poor lymphoproliferative responses have been described in cases of lepromatous leprosy, and uncertainty remains concerning the cause of this phenomenon, as to whether it is of pathogen or host induction, and whether it is due to failure of lymphocytes to respond, or of depressed APC capability. T-cell subset depletion experiments using MACS confirmed that the proliferative response to JPPD is effected by lymphocytes of $CD4^+$ phenotype, with reduced responses noted in samples depleted of this subset. DTH responses are cytokine-mediated, and are required for successful elimination of mycobacteria but frequently result in immunopathology. Quantification of IFNγ and IL-2 elaboration by lymphocytes isolated from cases of paratuberculosis confirmed higher levels of these two lymphokines in the tuberculoid group of animals, and again no significant differences were noted in the production of these important mediators of DTH between the lepromatous and control animals. In general, positive correlation was noted between lymphoproliferative responses and lymphokine elaboration for diseased animals. Differences were observed in levels of proliferation and cytokine elaboration between lymphocytes from the three tissues studied, with lowest levels noted in LPL. However, MLNL provided a good indication of local responses to *Map* infection. It was also observed that positive responses by lymphocytes of MLN and

ileal origin were reflected in positive responses by PBL, and that assay of circulating lymphocytes, while being more easily evaluated (and also being possible *ante mortem*), provided an indication of the presence of local responses. However, a positive response by PBL did not necessarily equate to a positive response by MLNL or LPL.

This study confirmed that groups of animals were therefore characterised by significant patterns of responses, with the tuberculoid group showing stronger proliferative responses and cytokine elaboration, and the group of lepromatous cases having poor proliferative and cytokine responses. Within the groups of infected animals, a minority of individual cases did not conform to the expected pattern, notably one animal of the lepromatous group (P40). This animal had strong proliferative and cytokine responses in the presence of high numbers of AFB and a marked macrophage infiltrate which was extensive and diffuse in character, suggesting that a range of immune responses may occur in individual animals within the classifications.

It was considered important to examine cytokine mRNA in the ileum of infected animals, and this was done by RT-PCR. It was hypothesised, in accordance with the Th1/Th2 paradigm, that differing profiles of cytokine mRNA expression might be found in the lesions of the different histological manifestations of paratuberculosis. Higher levels of cytokine mRNA were evident in the tissues of diseased animals, however the anticipated pattern of high IFNγ and IL-2 levels in the ileum of tuberculoid cases was not confirmed. Similarly, the levels of the type 2 cytokine IL-4 were found to be higher in the tuberculoid group of animals compared with the lepromatous group. These observations were in direct opposition to the hypothesised profiles. However, levels of IL-10 mRNA were noted to be highest in the tissues of the lepromatous group, and this is consistent with the predominantly macrophage

infiltrate, and may result in downregulation of CMI and promotion of an antibody response in this form of the disease. The gut is a complex organ with considerable immunological function, and the cytokine mRNA profiles may therefore not be readily comparable with those observed in other tissues, such as skin or peripheral blood.

In the study of a herd of goats, the inverse relationship between lymphocyte proliferation and serum antibody levels was clearly demonstrable for the entire herd, as were negative correlations between the AFB burden and lymphoproliferative response, and conversely, a positive relationship between AFB number and serum antibody in the group of goats which had lesions of paratuberculosis. As was noted for ovine cases, higher levels of circulating B cells were detected in goats with intestinal lesions. However, a number of species-specific differences were noted in goats, namely the paucity of gross lesions, the fact that lesions need not be present in the distal ileum, and the presence of caseous necrosis and calcification in this species.

As there is very little published data on the immunology of paratuberculosis in sheep and goats, this thesis is by definition of a preliminary nature. However, this study has provided a basis for further research, and considerable subsequent investigation into this significant disease of ruminants ought to be undertaken. It is likely that further studies would significantly increase our understanding of the pathogenesis of this and other mycobacterial conditions. For example, further examination of cell-mediated immune responses could be performed using experimentally infected animals, and may provide information on the progression of the disease from initial infection to clinical stages. This may elucidate the reason for the existence of the two distinctive types of lesion. Mycobacterial infections involve a balance between pathogenicity of the organism and host responses, and as such the host's immune responses are not static, but fluctuate during the course of the disease, the best example of this being the phenomenon of the reversal reaction in leprosy. It is

possible that similar reactions may occur in paratuberculosis, with tuberculoid-type lesions representing an upregulation of the host immune responses resulting in decreased AFB burdens. Alternatively, the high AFB burdens observed in cases with lepromatous lesions may be the result of diminished immune responses, or indeed the multiplication of the bacilli may directly result in downregulation of the host's cell-mediated responses. Preparation of autologous APCs, and experimental infection of APCs, would be possible in an experimental situation and may provide important data on antigen presentation and ensuing lymphocyte responses in mycobacterial disease. The development and availability of more highly characterised, specific *Map* antigens would further enhance understanding of the immune response, and permit delineation of antigen-specific T-cell clones, and therefore improve the ability to examine T-cell effector mechanisms. Studies employing *in-situ* hybridisation would allow determination of cytokine production by individual cells, and relation of this to cell phenotype, in the lesions of paratuberculosis. The existence of type 1- and type 2-like responses in ruminants is probable, but requires further clarification, and this should be possible with the development of additional reagents for these species. Examination of IL-12 may have particular relevance to paratuberculosis infection and its associated immunopathology. Elevated serum antibody levels have been recorded during the course of this study, but determination of *Map*-specific antibody isotypes was not performed. The relative predominance of antibody isotypes in leprosy patients has been used in the extrapolation of T-helper cell function (Ulrich *et al.,* 1995), and a similar study may provide information on the type of responses in the different forms of paratuberculosis.

In conclusion, this thesis has contributed to our understanding of paratuberculosis in sheep and goats, in particular to the relation of the differing pathological forms of the disease and the corresponding underlying immunology.

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APPENDICES

APPENDICES TO CHAPTER 3

Appendix 3.1: Reagents and buffers.

Phosphate-buffered saline (PBS).

0.15 M PBS, pH 7.2
Sodium chloride (9.00 gl^{-1}
Potassium chloride (0.20 gl^{-1})
0.008 M Disodium hydrogen phosphate, Na2HPO4 (1.15 g l^{-1})
Potassium dihydrogen phosphate (0.20 gl^{-1})

Dissolved in 1000 ml distilled water.

Prepared as x10 stock solution and diluted as required.
Sterilised by autoclaving and 20 mM (final concentration) sodium azide can be added as a preservative as required.

Glucose oxidase method for blocking of endogenous peroxidase activity.(Andrew and Jasani, 1987).

Stock solutions

β-D glucose	100mM [1.802 g 100 ml^{-1} PBS]
Glucose oxidase	100 units.1 ml^{-1} PBS
Na azide	10mM [0.065g 100 ml^{-1} PBS]
PBS	0.1M pH 7.3

Working solution

100mM β-D glucose	1 ml
10mM Na azide	1 ml
100 units.1ml PBS glucose oxidase	0.5 ml
0.1M PBS pH 7.3	7.9 ml

1. Working solution made up fresh, and warmed to 37°C for 20 min. before being applied to section.
2. Incubated on sections at 37°C.
3. Incubation time depends on the amount of endogenous peroxidase in the tissue. For gut, 1hour at 37°C was found to be optimal.

Appendix 3.2: Immunostaining procedure, using Vectorstain Elite ABC Kit.

1 Cut sections on cryotome at 6-7 μ thick.
2 Air dry at least 2 hours.
3 Fix in cold acetone for 5 min., and air dry.
4 Place in coverplates and wash in PBS x 3.
5 Block endogenous peroxidase staining with glucose oxidase for 1hr. at 37°C.
6 Wash in PBS x 3.
7 Block non specific binding with normal sera for 15 min. at RT.
8 Add primary monoclonal antibody and incubate overnight at 4°C.
9 Wash in PBS x 3.
10 Add second layer biotinylated antibody, 30 min. at RT.
11 Wash in PBS x 3.
12 Add streptavidin compound, 30 min. at RT.
13 Wash in PBS x 3.
14 Add chromogen (DAB), approx. 10 min. at RT.
15 Wash tapwater.
16 Counterstain in Meyer's Haematoxylin 1min. and blue in Scott's Tap Water Substitute.
17 Clear through graded ethanol and xylene, and mount in DPX.

Appendix 3.3: Totals of immunoperoxidase-stained IELs counted in 10 lengths of ileal epithelium.

Control				Lepromatous				Tuberculoid			
sheep	SBUT4	SBUT8	86D	sheep	SBUT4	SBUT8	86D	sheep	SBUT4	SBUT8	86D
1	0	13	11	P5	0	28	12	P8	1	78	6
2	1	13	19	P6	0	25	6	P19	0	47	30
3	0	24	6	P9	2	19	16	P23	0	42	15
4	0	17	21	P10	3	20	10	P25	0	90	51
5	2	18	15	P11	0	16	6	P27	0	49	28
6	2	16	8	P12	0	21	36	PC39	1	64	63
C3	0	13	12	P13	0	24	19				
C5	0	16	15	P14	0	17	13				
C24	0	22	24	P15	0	25	6				
C25	0	18	10	P16	0	33	8				
C29	0	31	36								

Appendix 3.4: Cell counts from immunoperoxidase-stained tissue sections. LP: lamina propria cells; EA: epithelial associated; 4: SBUT4; 8: SBUT8; γδ: 86D; v: villus cells; c: crypt cells

Control group.

sheep		LP				sum	EA				sum	LP+ EA				total
1	4v	57	65	53	59	234	0	0	0	0	0	57	65	53	59	234
	4c	19	20	23	16	78	0	0	0	0	0	19	20	23	16	78
	8v	36	45	44	52	177	30	39	30	32	131	66	84	74	84	308
	8c	14	17	15	13	59	0	5	2	7	14	14	22	17	20	73
	γδv	5	7	5	13	30	4	1	3	5	13	9	8	8	18	43
	γδc	2	2	6	6	16	1	1	4	2	8	3	3	10	8	24
2	4v	110	90	81	49	330	0	0	0	0	0	110	90	81	49	330
	4c	50	30	20	24	124	0	0	0	0	0	50	30	20	24	124
	8v	66	55	57	50	228	80	49	96	59	284	146	104	153	109	512
	8c	15	10	8	12	45	14	6	13	10	43	29	16	21	22	88
	γδv	9	11	4	10	34	9	7	4	5	25	18	18	8	15	59
	γδc	7	5	8	3	23	5	6	4	3	18	12	11	12	6	41
3	4v	39	56	57	77	229	0	0	0	0	0	39	56	57	77	229
	4c	13	9	12	8	42	0	0	0	0	0	13	9	12	8	42
	8v	36	22	47	45	150	36	47	44	47	174	72	69	91	92	324
	8c	7	9	5	12	33	9	8	5	8	30	16	17	10	20	63
	γδv	5	10	8	12	35	3	2	2	5	12	8	12	10	17	47
	γδc	4	5	5	4	18	1	2	2	1	6	5	7	7	5	24
4	4v	65	84	63	54	266	0	0	0	0	0	65	84	63	54	266
	4c	9	29	18	12	68	1	0	0	0	1	10	29	18	12	69
	8v	55	30	41	28	154	56	57	48	58	219	111	87	89	86	373
	8c	8	9	12	14	43	12	8	6	9	35	20	17	18	23	78
	γδv	3	3	6	8	20	14	6	4	5	29	17	9	10	13	49
	γδc	7	4	8	5	24	3	1	0	3	7	10	5	8	8	31
5	4v	114	78	96	81	369	2	0	4	0	6	116	78	100	81	375
	4c	17	42	23	29	111	0	1	0	0	1	17	43	23	29	112
	8v	51	60	108	96	315	90	95	107	119	411	141	155	215	215	726
	8c	25	26	30	20	101	21	20	8	5	54	46	46	38	25	155
	γδv	13	12	13	17	55	2	8	4	9	23	15	20	17	26	78
	γδc	6	4	6	13	29	7	5	5	6	23	13	9	11	19	52
6	4v	103	96	118	108	425	0	1	2	0	3	103	97	120	108	428
	4c	24	13	17	12	66	0	1	0	0	1	24	14	17	12	67
	8v	35	53	72	30	190	43	44	50	44	181	78	97	122	74	371
	8c	10	28	11	15	64	5	10	8	7	30	15	38	19	22	94
	γδv	6	6	7	11	30	15	6	7	8	36	21	12	14	19	66
	γδc	2	4	7	6	19	3	3	2	4	12	5	7	9	10	31

C7	4v	81	66	72	79	298	3	4	0	0	7	84	70	72	79	305
	4c	19	27	30	8	84	0	0	0	0	0	19	27	30	8	84
	8v	66	75	86	68	295	40	44	89	60	233	106	119	175	128	528
	8c	15	9	12	19	55	30	15	22	16	83	45	24	34	35	138
	γδv	30	21	36	24	111	5	5	9	9	28	35	26	45	33	139
	γδc	12	8	8	8	36	2	2	4	4	12	14	10	12	12	48
C24	4v	10	33	13	27	83	0	0	0	0	0	10	33	13	27	83
	4c	28	20	23	13	84	0	0	0	0	0	28	20	23	13	84
	8v	11	17	14	14	56	29	23	39	35	126	40	40	53	49	182
	8c	2	12	3	1	18	16	6	18	15	55	18	18	21	16	73
	γδv	7	2	4	5	18	12	8	9	7	36	19	10	13	12	54
	γδc	0	1	0	4	5	3	4	2	4	13	3	5	2	8	18
C25	4v	70	60	56	80	266	0	0	0	0	0	70	60	56	80	266
	4c	16	18	14	18	66	1	0	0	0	1	17	18	14	18	67
	8v	45	35	23	40	143	60	57	47	47	211	105	92	70	87	354
	8c	14	3	9	5	31	5	20	8	5	38	19	23	17	10	69
	γδv	11	9	12	13	45	12	12	14	19	57	23	21	26	32	102
	γδc	6	9	6	7	28	6	5	4	4	19	12	14	10	11	47
C29	4v	60	60	63	70	253	0	1	2	0	3	60	61	65	70	256
	4c	20	13	20	17	70	3	1	1	2	7	23	14	21	19	77
	8v	61	62	75	53	251	67	64	73	72	276	128	126	148	125	527
	8c	25	16	11	16	68	17	22	12	18	69	42	38	23	34	137
	γδv	8	8	15	16	47	12	16	10	12	50	20	24	25	28	97
	γδc	4	5	9	3	21	5	4	11	4	24	9	9	20	7	45
C3	4v	99	89	89	101	378	2	1	1	0	4	101	90	90	101	382
	4c	32	41	21	26	120	0	0	0	1	1	32	41	21	27	121
	8v	126	170	90	109	495	30	60	49	41	180	156	230	139	150	675
	8c	22	26	19	19	86	12	12	17	12	53	34	38	36	31	139
	γδv	13	17	14	13	57	8	7	8	4	27	21	24	22	17	84
	γδc	6	13	13	5	37	4	0	5	1	10	10	13	18	6	47
C5	4v	84	63	70	68	285	0	0	0	0	0	84	63	70	68	285
	4c	22	14	15	15	66	0	0	0	0	0	22	14	15	15	66
	8v	20	58	30	28	136	35	47	35	36	153	55	105	65	64	289
	8c	6	13	13	5	37	14	10	12	18	54	20	23	25	23	91
	γδv	6	3	8	13	30	6	3	7	6	22	12	6	15	19	52
	γδc	8	6	2	3	19	5	0	2	1	8	13	6	4	4	27

Lepromatous group.

		LP				sum	EA				sum	LP+ EA				total
P2	4v	81	37	33	56	207	2	1	0	0	3	83	38	33	56	210
	4c	35	47	33	27	142	0	0	0	0	0	35	47	33	27	142
	8v	70	25	19	35	149	52	35	13	48	148	122	60	32	83	297
	8c	20	35	25	26	106	40	42	30	17	129	60	77	55	43	235
	γδv	16	4	22	17	59	2	4	22	8	36	18	8	44	25	95
	γδc	17	17	10	13	57	10	3	3	5	21	27	20	13	18	78
P3	4v	27	31	45	56	159	0	4	8	4	16	27	35	53	60	175
	4c	26	39	21	35	121	1	0	0	1	2	27	39	21	36	123
	8v	10	6	6	10	32	9	9	7	22	47	19	15	13	32	79
	8c	13	6	10	18	47	3	3	0	0	6	16	9	10	18	53
	γδv	12	5	6	4	27	0	3	2	2	7	12	8	8	6	34
	γδc	6	6	4	8	24	1	0	3	1	5	7	6	7	9	29
P4	4v	80	58	70	47	255	4	3	0	1	8	84	61	70	48	263
	4c	14	15	13	23	65	0	2	0	1	3	14	17	13	24	68
	8v	35	17	18	15	85	5	16	8	14	43	40	33	26	29	128
	8c	4	4	8	4	20	4	6	4	4	18	8	10	12	8	38
	γδv	6	12	16	20	54	1	2	3	0	6	7	14	19	20	60
	γδc	0	4	3	1	8	3	0	1	0	4	3	4	4	1	12
P5	4v	39	49	31	65	184	0	0	0	1	1	39	49	31	66	185
	4c	15	10	18	9	52	0	0	1	1	2	15	10	19	10	54
	8v	25	24	27	13	89	21	27	26	39	113	46	51	53	52	202
	8c	6	6	5	7	24	11	5	6	6	28	17	11	11	13	52
	γδv	9	6	15	7	37	4	5	10	1	20	13	11	25	8	57
	γδc	6	5	4	4	19	3	1	2	1	7	9	6	6	5	26
P6	4v	29	38	25	12	104	0	0	2	0	2	29	38	27	12	106
	4c	10	13	15	20	58	1	2	0	0	3	11	15	15	20	61
	8v	8	44	33	15	100	31	55	35	40	161	39	99	68	55	261
	8c	10	7	8	19	44	11	14	16	18	59	21	21	24	37	103
	γδv	26	8	9	13	56	4	2	2	5	13	30	10	11	18	69
	γδc	9	6	7	5	27	1	5	0	1	7	10	11	7	6	34
P9	4v	47	44	51	48	190	2	1	0	0	3	49	45	51	48	193
	4c	14	16	21	18	69	0	0	0	0	0	14	16	21	18	69
	8v	21	28	21	50	120	30	31	32	35	128	51	59	53	85	248
	8c	10	10	13	20	53	10	8	12	7	37	20	18	25	27	90
	γδv	5	5	8	4	22	8	5	7	1	21	13	10	15	5	43
	γδc	5	1	1	1	8	2	3	0	2	7	7	4	1	3	15

P10	4v	39	58	30	66	193	0	2	0	0	2	39	60	30	66	195
	4c	26	28	16	20	90	0	0	0	0	0	26	28	16	20	90
	8v	31	52	49	66	198	45	43	39	31	158	76	95	88	97	356
	8c	5	9	11	4	29	7	4	11	7	29	12	13	22	11	58
	γδv	7	14	9	13	43	9	6	7	9	31	16	20	16	22	74
	γδc	4	10	3	4	21	3	3	2	4	12	7	13	5	8	33
P11	4v	43	10	29	25	107	0	2	3	0	5	43	12	32	25	112
	4c	14	18	18	19	69	0	1	0	0	1	14	19	18	19	70
	8v	41	10	19	5	75	11	23	38	35	107	52	33	57	40	182
	8c	18	6	8	7	39	4	8	14	5	31	22	14	22	12	70
	γδv	24	13	5	13	55	21	3	13	1	38	45	16	18	14	93
	γδc	5	4	5	8	22	4	6	5	2	17	9	10	10	10	39
P13	4v	61	51	49	35	226	1	0	1	1	3	62	52	49	36	229
	4c	42	18	27	22	109	0	0	0	0	0	42	18	27	22	109
	8v	35	35	20	45	135	26	25	49	55	145	61	60	69	90	280
	8c	17	13	14	19	63	0	13	8	12	33	17	26	22	31	96
	γδv	25	14	19	10	68	2	16	5	2	25	27	30	24	12	93
	γδc	6	7	9	6	28	3	4	1	0	8	9	11	10	6	36
P14	4v	33	34	27	24	118	0	0	0	0	0	33	34	27	24	118
	4c	20	18	17	10	65	1	0	1	0	2	21	18	18	10	67
	8v	22	28	22	20	92	16	24	20	21	81	38	52	42	41	173
	8c	17	8	25	19	69	3	12	2	8	25	20	20	27	27	94
	γδv	16	39	13	8	76	4	16	13	9	42	20	55	26	17	118
	γδc	6	11	9	10	36	7	0	5	6	18	13	11	14	16	54
P15	4v	18	21	21	48	108	0	0	0	0	0	18	21	21	48	108
	4c	21	23	15	12	71	0	0	0	0	0	21	23	15	12	71
	8v	8	25	16	16	65	14	20	27	30	91	22	45	43	46	156
	8c	27	18	18	15	78	7	15	5	11	38	34	33	23	26	116
	γδv	11	6	6	5	28	6	2	3	8	19	17	8	9	13	47
	γδc	4	2	4	3	13	0	3	2	1	6	4	5	6	4	19
P16	4v	20	26	34	11	91	0	0	1	1	2	20	26	35	12	93
	4c	5	11	9	7	32	0	0	0	0	0	5	11	9	7	32
	8v	15	25	34	32	106	40	41	43	25	149	55	66	77	57	255
	8c	7	9	6	5	27	8	6	10	6	30	15	15	16	11	57
	γδv	20	12	8	7	47	6	4	2	5	17	26	16	10	12	64
	γδc	4	5	2	2	13	0	0	0	2	2	4	5	2	4	15

Tuberculoid group

		LP				sum	EA				sum	LP+ EA				total
P25	4v	123	160	146	196	625	0	0	0	1	1	123	160	146	197	626
	4c	45	79	66	64	254	0	0	0	0	0	45	79	66	64	254
	8v	77	55	76	63	271	83	54	68	74	279	160	109	144	137	550
	8c	48	37	71	27	183	22	21	11	14	68	70	58	82	41	251
	γδv	38	30	31	21	120	19	18	31	28	96	57	48	62	49	216
	γδc	10	4	15	10	39	4	6	4	5	19	14	10	19	15	58
P27	4v	92	119	139	126	476	0	0	1	2	3	92	119	140	128	479
	4c	89	77	88	81	335	3	2	0	0	5	92	79	88	81	340
	8v	43	80	82	96	301	46	23	29	62	160	89	103	111	158	461
	8c	46	43	29	42	160	14	5	9	9	37	60	48	38	51	197
	γδv	20	17	14	22	73	6	8	7	1	22	26	25	21	23	95
	γδc	18	10	12	16	56	3	1	1	7	12	21	11	13	23	68
P23	4v	103	77	85	96	361	0	0	0	0	0	103	77	85	96	361
	4c	58	90	80	94	322	0	0	0	1	1	58	90	80	95	323
	8v	55	71	54	51	231	15	20	14	22	71	70	91	68	73	302
	8c	16	17	20	25	78	9	12	7	10	38	25	29	27	35	116
	γδv	22	26	10	20	78	8	4	3	2	17	30	30	13	22	95
	γδc	10	8	15	1	34	2	3	0	0	5	12	11	15	1	39
P19	4v	86	96	88	126	396	0	2	0	0	2	86	98	88	126	398
	4c	110	59	60	44	273	1	0	0	0	1	111	59	60	44	274
	8v	43	35	38	54	170	37	27	35	32	131	80	62	73	86	301
	8c	25	23	41	15	104	7	13	10	3	33	32	36	51	18	137
	γδv	16	29	23	25	93	1	7	4	15	27	17	36	27	40	120
	γδc	9	14	9	15	47	1	3	0	0	4	10	17	9	15	51
PC39	4v	119	76	81	113	389	2	3	3	0	8	121	79	84	113	397
	4c	19	27	24	33	103	0	0	2	0	2	19	27	26	33	105
	8v	56	48	44	70	218	144	122	80	92	438	200	170	124	162	656
	8c	26	25	18	21	90	17	27	13	12	69	43	52	31	33	159
	γδv	21	14	17	14	66	19	23	18	28	88	40	37	35	42	154
	γδc	9	9	4	11	33	2	10	7	9	28	11	19	11	20	61
P8	4v	95	137	91	125	448	0	0	0	0	0	95	137	91	125	448
	4c	69	60	65	51	245	0	0	0	0	0	69	60	65	51	245
	8v	52	69	68	69	258	42	68	70	54	234	94	137	138	123	492
	8c	45	20	31	24	120	0	3	9	1	13	45	23	40	25	133
	γδv	14	19	8	13	54	5	5	2	0	12	19	24	10	13	66
	γδc	9	11	5	6	31	2	1	1	0	4	11	12	6	6	35

APPENDICES TO CHAPTER 4

Appendix 4.1: Reagents and buffers

PBA
Phosphate Buffered Saline with:

1%	Bovine Serum Albumin (Sigma)
20 U ml^{-1}	Heparin (Leo Laboratories, Princes Risborough, UK)
0.1%	Sodium Azide (Sigma)

1% paraformaldehyde

100 ml	PBS
1 g	paraformaldehyde

Adjusted to pH 7.2

Appendix 4.2: FACS analysis of IEL (cells isolated from EDTA digestion). Figures are percentages of positively stained cells. Listed in order of control (C), lepromatous (P) and tuberculoid cases (suffix T).

Sheep	SBUT4	SBUT8	86D	CC15	BCELL	VPM19	SW73.2	VPM36	VPM54	ST1
C38	14.82	43.48	6.70	5.16	21.06	87.96				
C40	26.45	41.43								
C41	5.33	78.75	8.91	17.78	0.52	92.86				
C42	11.09	76.73	11.15	0.46	2.16	94.21				5.40
C43	25.38	16.46	10.98							
C44	14.23	20.19	4.54	3.86	30.47	78.97		54.02	70.53	
C45	11.36	81.14	7.50	7.38		97.59				14.16
C46	14.06	56.54	10.97	7.39	8.98	93.14				11.55
C47	14.41	53.66	14.82	8.55		95.09				13.18
C48	5.02	72.03	7.20	7.64		94.82		27.76	86.17	12.30
C49	6.40	66.40	7.53	3.81	6.12	96.06			49.21	4.83
C50	9.59	20.08	12.65	5.60	65.36	93.12		44.36	60.75	
C51	8.64	73.93	6.83	5.87	2.60	97.78			64.38	
C52	8.34	64.87	10.65	3.23	3.04	96.60	92.15	20.17	72.57	9.67
C53	8.26	41.64	9.27	5.74	38.21	89.50	79.79	25.35	49.70	7.79
C54	3.49	75.18	7.82	5.05	22.37	99.53	99.35	75.22	89.12	4.12
P24	9.42	0.47	5.71							
P22	15.91	49.10	4.50	4.10		73.23				
P30	10.41	13.75	3.52	2.33		72.45	52.70			11.77
P31	7.93	78.68	9.58	6.09		97.71	90.00		61.79	7.96
P32										
P33	5.99	38.21	11.11	5.19	6.80	79.06	88.20	28.74	68.28	5.68
P35	17.21	56.41	5.89	4.59	3.56	91.67	76.92			17.82
P36	20.84	41.39	12.65	11.42	24.31	97.35	92.71	45.82	71.03	21.75
P37	19.85	35.82	9.67	7.53	19.68	93.45	84.76	54.03	68.45	20.71
P38	12.82	48.48	4.36	2.96	23.69	88.17	64.09	28.12	29.02	12.28
P39	9.52	69.91	10.25	11.74	2.93	87.27	95.48	75.51	73.70	9.61
P40	21.52	4.56	6.03	5.58	52.95	87.70	61.82	57.33	59.28	24.16
P25T	33.03	19.90	3.86	0.57		88.71		52.92	37.34	28.93
P27T	11.21	66.03	4.05	2.57		98.78		34.61	42.78	11.33
P23T	18.32	12.01	1.75	2.39						
P29T	29.27	42.79	5.05	4.73	10.85	94.82	87.70	54.66	70.76	28.82
P34T	21.33	58.03	5.58	2.71	23.67	88.18	96.18	26.07	39.16	16.43
P41T	57.26	9.03	5.45	5.05	2.39	94.15	85.35			

Appendix 4.3: FACS analysis of LPL. Figures are percentages of positively stained cells. Listed in order of control (C), lepromatous (P) and tuberculoid cases (suffix T).

Sheep	SBUT4	SBUT8	86D	CC15	BCELL	VPM19	SW73,2	VPM36	VPM54	ST1
C38	50.99	25.23	3.30	3.46	11.45	91.12				
C41	61.37	33.43	6.94	3.45	24.52	95.06				61.00
C42	44.57	34.47	10.32	1.11	19.85	97.09				44.36
C43	56.16	18.91	10.41	0.53	1.97	91.95				57.95
C44	57.39	23.28	4.63	4.85	15.87	94.48				56.38
C45	57.60	39.26	5.05	7.47	6.36	99.35				58.08
C46	59.64	28.54	8.11	10.85	1.01	98.00				58.69
C47	62.71	24.44	9.51	10.63	0.97	95.85				51.49
C48	28.23	46.85	6.67	9.00	0.52	94.41			70.29	27.43
C49	47.67	30.91	4.04		5.19	94.09				46.10
C50	17.59	4.91	1.42	1.83	15.19	60.84		21.85	32.37	
C51	64.17	25.33	5.45	6.85	3.07	97.92				
C52	35.58	24.41	2.51	2.31	6.23	78.25	68.64		41.58	33.00
C53	40.27	10.01	4.63	3.13	5.29	75.64	56.77			44.87
C54	9.51	33.36	10.16	4.24	1.32	98.42	98.54	57.87	79.80	11.60
P24	44.68	41.86	15.80		2.19	51.60		44.06		
P22	25.00	49.48	6.03	15.58	7.85	44.56				
P30	34.16	26.77	6.98	4.87	6.55	89.70	65.84	20.32	39.90	31.64
P31	46.97	26.94	7.09	5.65	4.59	93.37	80.73	54.35	72.35	46.70
P32										
P33	34.03	27.87	3.86	13.74	2.83	90.43	71.17	35.69	34.05	7.67
P35	36.48	43.71	5.83	6.00	1.27	93.85	90.14	36.78	47.66	36.38
P36	45.93	28.31	13.17	13.12	11.25	97.01	85.50	65.21	62.00	46.37
P37	51.85	25.13	7.58	10.23	7.19	90.01	78.95	60.94	62.28	51.55
P38	45.64	34.00	6.71	6.85	2.38	95.18	86.50	84.34	75.40	47.33
P39	32.37	40.10	7.37	6.52	2.35	90.53	88.64	62.42	51.81	32.92
P40	35.07	6.90	7.59	6.80	4.41	78.42	60.06		35.32	34.50
PC39	34.35	30.94	8.14	2.08	6.08	70.00			37.96	32.75
T										
P25T	66.15	15.97	2.67	0.98	2.96	92.17		33.42	22.53	57.25
P27T	60.07	24.44	3.93	3.87	1.84	96.15			58.04	58.78
P23T	20.76	16.73	4.75	5.29	6.46	54.09				
P29T	61.48	19.44	3.43	3.98	1.59	90.68	88.98	59.85	64.92	63.32
P34T	60.80	17.46	5.22	4.44	2.37	93.82	87.14	27.87	66.14	58.42
P41T	46.46	11.92	11.03	11.21	8.89	96.10	83.79	78.97	75.01	47.13

Appendix 4.4: FACS analysis of MLNL. Figures are percentages of positively stained cells. Listed in order of control (C), lepromatous (P) and tuberculoid cases (suffix T).

Sheep	SBUT4	SBUT8	86D	CC15	BCELL	VPM19	SW73,2	VPM36	VPM54	ST1
C38										
C41	31.24	8.43	2.43	0.51	47.85	86.89		64.90	52.53	32.62
C42	19.92	19.60	2.33	0.86	44.26	96.25		68.32	71.39	18.50
C43	32.12	12.83	2.94	1.35	35.08	96.50		48.89	71.65	31.14
C44	23.69	9.47	1.03	1.47	60.31	97.48		68.18	75.00	23.99
C45	38.75	23.79	1.59	2.48	32.55	98.56		44.29	58.69	37.75
C46	30.20	15.90	6.61	3.08	24.21	91.75		44.94	42.98	
C47										
C48	25.33	26.84	4.30	6.16	33.60	94.54		52.39	80.85	23.71
C49	23.21	20.26	2.31	3.13	45.64	97.92		61.89	81.18	22.57
C50	14.58	8.92	2.65	2.87	64.53	95.46		77.04	91.84	
C51	22.91	9.24	0.95	9.33	57.22	93.68		65.82	75.12	
C52	37.35	13.74	2.62	2.29	45.62	97.98	87.01	63.71	83.85	34.27
C53	17.64	14.74	2.20	2.43	53.82	93.50	92.04	67.28	72.81	17.39
C54	40.79	22.93	3.46	2.91	24.09	99.33	93.96	87.54	79.50	45.85
P24										
P30	28.99	11.55	8.52	2.54	39.82	96.54	87.68	72.45	70.39	29.92
P31	19.47	29.52	3.31	3.50	35.44	91.67	84.26	56.96	70.63	
P32	28.02	12.30		9.04						29.02
P33	33.62	13.75	5.65	4.63	25.92	96.29	81.12	47.17	47.17	5.37
P35	18.26	9.49	0.94	1.64	58.28	84.87	87.17	50.51	68.62	11.91
P36	28.43	26.82	6.03	6.41	35.96	99.21	90.76	75.57	77.78	27.73
P37	30.45	22.26	4.70	5.77	41.96	97.78	87.32	67.90	77.81	31.06
P38	21.83	12.54	2.93	2.69	51.71	89.27	86.46	72.57	74.81	25.30
P39	29.14	25.41	9.83	10.16	23.28	94.82	85.90	51.95	54.07	29.48
P40	21.89	6.85	3.71	3.52	62.09	98.10	71.64	65.56	65.50	24.51
P25T	26.98	39.92	5.00	0.76	20.64	94.98		44.32	39.73	26.32
P27T	21.73	15.16	2.94	3.64	40.58	94.06		49.60	74.79	21.42
P28T	22.04	8.79	5.37	7.84						16.52
P29T	20.58	12.83	2.02	2.50	48.54	92.12	86.20	59.38	67.56	20.08
P34T	32.96	15.62	2.83	2.71	30.57	88.90	89.05	47.31	64.20	16.65
P41T	22.28	8.07	2.83	3.08	62.41	96.65	87.01	74.82	76.90	23.16

Appendix 4.5: FACS analysis of PBL. Figures are percentages of positively stained cells. Listed in order of control (C), lepromatous (P) and tuberculoid cases (suffix T).

Sheep	SBUT4	SBUT8	86D	CC15	BCELL	VPM19	SW73,2	VPM36	VPM54	ST1
C38	6.04	11.65	10.53	10.47	43.54	99.53	62.00			
C41	7.19	16.12	7.54	1.55	31.92	97.85		53.71	48.46	7.16
C42	16.12	27.31	8.45	0.82		96.59		46.16	49.95	15.49
C43	13.60	9.75	9.22	1.28	19.16	91.60		40.52	66.99	12.65
C44	17.03	18.05	13.13	9.62	22.33	95.70		55.60	51.28	15.72
C45	9.67	35.78	7.19	8.08	26.37	97.42		56.01	67.54	9.65
C46	19.37	15.65	7.19	9.82	13.02	98.76		41.00	54.03	18.37
C47	8.63	10.44	13.63	11.67		90.64		47.42	46.03	5.31
C48	8.67	23.75	6.67	6.92	23.54	98.39		59.56	72.23	8.67
C49	12.43	31.51	8.32	7.63	26.71	98.00		45.32	61.72	12.64
C50	9.92	43.14	6.12	6.78	33.07	98.87		30.93	50.71	
C51	15.51	20.58	4.54	5.62	58.10	99.01		51.03	57.57	
C52	5.88	10.93	8.45	8.75	72.54	97.26	89.90	72.46	88.07	5.70
C53	14.05	23.92	5.45	6.90	51.42	92.25	82.31	28.72	64.87	10.66
C54	10.62	16.35	6.06	6.12	65.49	82.45	83.54	64.35	66.42	11.54
C55	11.75	6.04	15.52	15.22	62.67	99.66	78.39	68.56	63.47	
C56	23.07	17.48	5.83	5.29	46.58	96.85	66.75	65.26	48.14	
P24	20.33	19.42	3.06		26.14					
P30	9.21	6.37	1.50	4.32	78.58	98.44	89.62	76.00	78.45	9.71
P31	7.04	13.33	4.51	4.37	48.67	94.17	63.85	34.85	49.04	6.69
P32	25.60	14.24		8.83	14.51					18.17
P33	19.76	10.08	2.10		50.46	96.71	62.33	57.64		
P35	12.52	7.54	4.25	5.12	53.48	98.96	76.90	58.89	61.11	11.97
P36	5.20	9.87	5.28	7.19	65.46	98.15	92.40	82.37	89.64	3.85
P37	4.79	8.00	3.47	4.79	85.76	96.85	93.34	88.73	91.12	4.37
P38	4.00	8.67	1.26	3.13	70.60	94.04	83.40	78.17	80.43	3.45
P39	3.20	2.83	1.37	2.94	45.22	98.59	89.90	72.29	87.72	3.85
P40	3.91	3.28	1.90	6.06	78.09	97.31	88.99	89.38	87.81	3.71
P43	10.17	6.08	5.29		68.93	99.33	91.28			
P45	13.16	8.51	3.10	3.37	66.90	98.32	77.12	67.07	67.34	
P46	15.62	7.46	11.49	11.16	49.59	96.89	75.73	59.00	56.01	
P25T	15.72	16.42	4.24	2.46		92.67		49.27	42.27	14.54
P26T	15.74	8.35	1.43	1.29		95.57		48.53	62.06	15.00
P27T	2.28	6.55	2.91	3.33	32.71	99.26	27.46	74.96	87.62	3.28
P29T	21.98	13.71	6.21	7.79	40.84	93.62	67.06	31.80	60.22	23.17
P34T	16.17	11.83	9.08	13.60	50.26	98.74	70.46	73.37		20.07
P41T	4.04	5.08	1.89	3.04	88.78	99.12	93.76	89.29	91.25	3.96
P42T	15.51	7.46	2.60		76.75	97.67	92.01			
P44T	10.78	4.51	2.97	3.56	73.56	99.09	87.90	83.62	81.39	
P47T	10.92	13.63	2.92	3.47	54.92	79.15	88.53	78.21	74.65	

APPENDIX TO CHAPTER 6

Appendix 6.1: LSA SI values for individual animals, unsorted and depleted cells

MAb	*Animal*	*Unsorted*	*Depleted*	*Difference*
SBUT4	Sheep P42	61.0	6.19	-
	P43	2.60	2.31	-
	P44	9.39	0.92	-
	P47	5.28	2.16	-
	Goat A062	1.20	1.27	+
	A072	4.05	2.74	-
	A058	1.06	1.98	+
	A054	2.13	0.69	-
	A056	6.25	1.00	-
	A078	1.39	1.38	-
SBUT8	Sheep P44	2.93	7.77	+
	P46	11.40	3.11	-
	P47	6.17	23.78	+
	Goat A062	3.76	3.67	-
	A072	1.68	2.10	+
	A058	1.99	1.95	-
	A054	2.71	1.08	-
	A056	1.11	1.47	+
	A078	4.48	2.75	-
86D	Sheep P44	1.49	18.20	+
	P46	9.06	16.38	+
	P47	7.18	5.54	-
	Goat A060	0.66	1.23	+
	A073	2.23	1.69	-
	A069	3.63	2.50	-
	A062	1.39	1.59	+
	A072	4.02	3.20	-
	A058	1.41	2.94	+
	A054	2.17	2.52	+
	A056	1.65	3.34	+
	AO78	3.10	3.03	-

APPENDIX TO CHAPTER 8

Appendix 8.1: Reagents and buffers

TAE buffer (0.04M tris-acetate, 0.001M EDTA)

242 g	tris base
57.1 ml	glacial acetic acid
100 ml	0.5M EDTA (pH 8.0)

Made up to one litre in distilled water.

x20 SSPE buffer

175.3 g	NaCl	
27.6 g	$NaH_2PO_4.H_20$	(Equivalent to 31.2g of $NaH_2PO_4.2H_20$)
7.4 g	EDTA	

Made up to 800 ml with distilled water.
Adjust pH to 7.4 with 10N NaOH.
Adjust the volume to 1000 ml with distilled water and sterilise by autoclaving.

Hybridisation buffer (per 10 ml)

8.46 ml	distilled H_2O
1 g	dextran sulphate
1 ml	10% SDS

Incubate for 30 minutes at 65°C, before adding:

0.58 g	NaCl
0.4 ml	x20 SSPE

Incubate for 15 minutes at 65°C prior to use.

TRIS-EDTA Buffer

10mM TRIS-HCl	(pH 7.4)
1mM EDTA	(pH 8.0)

Radiolabelling mix

2.0 µl	10x PNK buffer
1.0 µl	oligonucleotide (at a concentration of 50ng l^{-1})
14.0 µl	RNAase free water
2.0 µl	^{32}P labelled ATP (equivalent to 20 µCi)
1.0 µl	PNK enzyme

APPENDIX TO CHAPTER 9

Appendix 9.1: FACS data for goats. Values are percentages of positively stained cells.

Tissue	Goat	SBUT4	SBUT8	86D	CC15	VPM8	VPM19	SW73,2	VPM 36	VPM 54	ST1
LPL	A080	48.65	30.17	7.52	6.45	14.32	96.93	83.76	36.39	25.69	48.48
	A063	63.56	26.80	6.85	6.42	1.82	98.79	81.54	61.83	35.85	63.40
	A052	60.30	29.03	4.32	5.19	5.54	96.09	81.95	60.26	65.23	60.20
	A053	34.78	42.18	5.31	5.42	7.04					
IEL	A080	23.23	46.28	6.15	7.00	21.24	96.65	79.17	49.53	41.03	22.62
	A063	28.31	64.00	8.64	6.50	3.04	98.44	86.56	10.33	30.95	27.16
	A052										
	A053	22.32	49.99	8.35	5.82	1.47	94.15	85.68	34.39	54.53	21.64
MLNL	A080	22.00	17.41	3.92	3.81	63.97	98.68	78.36	78.84	65.03	25.14
	A063	24.35	34.31	2.68	2.61	51.53	97.57	64.37	55.62	51.75	24.66
	A052	11.32	22.14	2.48	2.46	64.75	98.39	84.15	71.32	66.71	13.20
	A053	18.44	33.21	2.81	2.92	53.97	91.96	87.03	70.74	68.51	15.07
PBL	A078	11.23	15.67	6.00	7.25	54.94	98.95	69.63	65.21	62.94	
	A056	14.03	24.07	8.83	9.85	50.90	99.14	65.36	50.62	46.60	
	A072	22.01	28.94	6.23	8.67	33.07	99.18	43.65	43.57	41.56	
	A062	25.11	15.82	8.92	11.43	35.21	99.79	50.71	54.26	43.17	
	A058	16.53	10.60	3.46	3.96	35.39	87.57	77.32	49.70	64.73	
	A054	20.54	12.50	5.14	7.04	27.82	91.18	53.69	38.44	51.23	
	A073	16.61	9.46	12.90	13.33	57.68	99.39	63.92	65.28	63.04	
	A069	18.57	30.88	4.67	6.00	46.75	98.82	50.99	43.03	42.89	
	A059	13.27	20.46	12.65	13.71	58.63	98.79	58.35	53.68	53.00	
	A060	10.00	9.59	8.03	8.54	72.59	99.35	74.81	74.73	73.28	
	A066	10.60	47.88	2.60	3.87	64.20	96.95	72.70	64.21	65.53	
	A080	10.22	17.83	7.90	9.75	66.26	99.31	75.09	74.55	67.82	11.61
	A063	18.80	17.10	4.42	5.20	58.75	98.70	61.72	65.53	62.39	19.12
	A052	9.52	41.13	6.49	7.92	45.85	99.53	63.22	55.80	54.03	9.42
	A053	7.56	12.83	2.71	4.56	80.71	99.50	86.06	77.45	82.48	6.14
Lymph	A078	68.25	15.40	1.21		2.71	98.70	38.10			
	A056	61.57	13.42	2.83		13.80	99.31	47.04			
	A066	70.75	18.46	1.42	3.02	3.95	91.87	39.29	37.53	25.08	
	A060	57.24	20.30	2.16	2.87	21.73	99.37	40.85	38.37	31.50	
	A069	69.87	11.79	0.97	2.41	17.01	99.33	23.85	19.73	17.57	
	A073	63.04	13.33	1.52	2.00	32.37	99.70	42.02	30.37	28.72	
	A058	65.93	13.10	2.51	2.74		39.17	30.28	11.55	13.30	

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The Pathology of Ovine Paratuberculosis: Gross and Histological Changes in the Intestine and Other Tissues

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Summary

In sheep clinically affected with paratuberculosis, two distinct forms of microscopical pathology were recognized, related to a high or a low degree of mycobacterial colonization ("multibacillary" or "paucibacillary" presence). These forms were characterized by different types of cellular infiltrate in the ileal mucosa and submucosa. Statistical analysis demonstrated strong correlations between the presence of large numbers of acid-fast organisms and macrophage infiltration, and between small numbers and lymphocyte infiltration. Correlations also existed between high numbers of acid-fast bacteria and a positive serum antibody test result; and between the presence of giant cells and lymphocytes in the gut. This study suggests that in ovine paratuberculosis the same clinical and gross pathological changes can result from different pathogenetic mechanisms.

Introduction

Ruminant paratuberculosis (Johne's disease) is characterized by a chronic granulomatous enteritis and regional lymphadenitis caused by *Mycobacterium avium* subspecies *paratuberculosis* (*M. a. paratuberculosis*). Infection usually occurs soon after birth and, after a protracted incubation period, progressive weight loss and death occur in adulthood. The gross and histopathological changes in natural and experimental ovine paratuberculosis have been documented by authors in several countries (Stamp and Watt, 1954; Rajya and Singh, 1961; Nisbet *et al.*, 1962; Reddy *et al.*, 1984; Carrigan and Seaman, 1990; Perez *et al.*, 1992), sometimes with clinical and serological data. In particular, cases have been grouped on the basis of intestinal histological lesions, and differences in the types of cells infiltrating the gut (predominantly epithelioid cells or lymphocytes) have been recognized and related to the degree of mycobacterial infection in the tissues. It is assumed that the case groups represent different pathogenetic processes of the same disease, with different cellular populations playing a role in the production of chronic enteritis. However, the possible relationship of different cellular populations to clinical signs, serological findings, gross lesions and histological changes has not been

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evaluated statistically. The study reported here was designed to make good some of these gaps in knowledge.

Materials and Methods

Sheep

Adult female Scottish Blackface and Cheviot sheep (29 healthy and 45 diseased) were obtained from farms in southern Scotland. Diseased animals were selected from flocks with established clinical, pathological and microbiological evidence of paratuberculosis. No animal had received paratuberculosis vaccine. All sheep were graded for bodily condition on a scale of 1 (cachectic) to 5 (very fat), examined clinically and tested for serological evidence of infection by the agar gel immunodiffusion (AGID) test for paratuberculosis (Sherman *et al.*, 1984).

Gross Pathology and Sampling

Sheep were killed by intravenous barbiturate administration and subjected to a full necropsy in which all gross lesions of the intestine and other tissues were noted. Sheep with significant intercurrent disease were excluded from the study. Fresh intestinal and other tissue samples were fixed in buffered formol saline and processed by routine methods. Sections were stained with haematoxylin and eosin (HE), and by the Ziehl-Neelsen (ZN) method to detect acid-fast bacteria. The same necropsy and sampling procedure was used for each animal and intestinal samples were always taken within 10–15 min of death. Samples (2 × 2 cm) of the proximal duodenum (5 cm from the pylorus), mid-jejunum, terminal ileum (5 and 10 cm from the ileocaecal valve), caecum (opposite the ileocaecal valve), mid-spiral colon and terminal rectum (10 cm from the anus) were taken and lowered gently into fixative to preserve morphology. In addition, samples were taken from mesenteric, ileocaecal and prescapular lymph nodes, spleen, liver, kidney, myocardium, lung, uterus and mammary gland, and from the placenta and fetus in pregnant animals. Fresh samples of ileum for nucleic acid extraction were snap frozen in dry ice and isopentane and stored at −70°C.

Histopathology

Detailed histological assessments of the sections of control and diseased ileum were made by one observer (CJC) by grading from 0 (no lesions) to 3 (severe and extensive cellular infiltration), as described by Carrigan and Seaman (1990). The density and type of cellular infiltrate (lymphocytes, macrophages, neutrophil polymorphs, eosinophils and giant cells) were assessed in the mucosal lamina propria, submucosa and serosa. The percentage of macrophages infected with acid-fast bacteria was graded as 0 (0%), 1 (<20%), 2 (20–75%) or 3 (>75%) from an examination of a total of 200 macrophages in eight randomly selected high-power fields of mucosal lamina propria (four fields from each of two different sections). The same cells were used to determine the mean number of bacteria per macrophage, graded as 0 (none), 1 (1–10), 2 (10–60) or 3 (>60). Sheep graded 2 or 3 in both these categories were classified as "multibacillary" and those graded 0 to 1 were classed as "paucibacillary". This distinction then formed the basis of examining other data related to each sheep. The macrophage infiltration in the intestine was classified as diffuse and extensive (D), multifocal small granulomata (F), or occasional scattered cells (S).

Histological examination of other intestinal regions, mesenteric and ileocaecal lymph nodes, spleen, liver, kidney, myocardium, lung, uterus and mammary gland, and of the placenta and fetus in pregnant animals was performed, particular attention being paid to the presence of macrophages, granulomata and acid-fast organisms. Lesions were graded 0–3 as above.

Detection of M. a. paratuberculosis *by the Polymerase Chain Reaction*

Specific *M. a. paratuberculosis* DNA was detected, after extraction of genomic DNA from frozen samples of ileum, by the Bioline InVisorb50 (Bioline, London, UK) protocol. Briefly, tissue was ground and incubated with lysis buffer and carrier suspension. After centrifugation the pellet was washed and resuspended in elution buffer and DNA was eluted twice at 52°C. Oligonucleotide primers specific for the IS-900 DNA sequence of *M. a. paratuberculosis* (1932, 5′ GCG GGA TCC GTG GCA CAA CCT GTC TG and 1933, 5′ GCG GGA TCC TCA AGC CGC CGC GGT AG) were used in the polymerase chain reaction (PCR) with sample DNA, positive or negative controls, and buffer containing deoxynucleotide triphosphates (dATP, dGTP, dCTP, dTT), detergent and Taq polymerase (Vary *et al.*, 1990). After 33 cycles (denaturation at 95°C for 45 s, annealing at 55°C for 45 s, primers extension at 72°C for 2 min) and a final extension period of 5 min, PCR products were loaded on to a 2% agarose gel for electrophoresis. Gels were stained with ethidium bromide to reveal positive PCR products of 1200 bp size.

Statistical Analysis

Histological scoring data were found to be normally distributed within groups. Initial comparisons among the three identified groups of sheep (see Results) were tested by analysis of variance test with subsequent comparisons between two groups made by Student's *t*-test, $P<0{\cdot}05$ being considered significant. Correlations between groups were tested by the covariance of the two data sets divided by the product of their standard deviations (data analysis programme, Microsoft Excel).

Results

Clinical and Diagnostic Findings

Diseased animals were initially selected on clinical criteria, including chronic weight loss, flock history, and sometimes a positive serum AGID test. However, further classification was made on the basis of the histological findings and the PCR results (see below). The findings in diseased animals later classified as "multibacillary" or "paucibacillary" are summarized in Table 1. (Both "bacillary" types were recognized within individual flocks.) All paratuberculous sheep were in poor condition and some were very ill at the time of euthanasia. Faeces consisted of pellets in most cases but some animals had soft pasty faeces (multibacillary 16%, paucibacillary 14%) and 19% of multibacillary sheep had diarrhoea. The AGID test was positive in 88% of multibacillary sheep but in only 36% of paucibacillary animals (72% of all diseased animals). All control animals were in good condition, with firm, pelleted faeces, and gave a negative AGID test result.

Necropsy and Gross Lesions

Data are summarized in Table 1. Most carcasses in the diseased groups (multibacillary and paucibacillary) were emaciated, with some degree of subcutaneous oedema. Within the multibacillary group all sheep had markedly and diffusely thickened ileal walls, with dilated intestinal lumina. Mucosal surfaces had a granular appearance and in 90% of cases they showed pigmentation ranging from light yellow to deep orange. Prominent transverse

Table 1
Clinical findings and gross pathology

Observation	*Results* in*		
	control sheep (n = 29)	*multibacillary sheep (n = 31)*	*paucibacillary sheep (n = 14)*
Condition score (mean)	3·06	1·10	1·30
Faeces—pelleted	100	65	86
—soft	0	16	14
—diarrhoea	0	19	0
AGID test + ve	0	88	36
Necropsy:			
Emaciation	0	100	85
Carcase oedema	0	83	75
Ileum—thickened wall	0	100	85
—pigmented mucosa	0	90	0
—mucosal ridging	0	43	31
—mucosal crevicing	0	26	8
—lymphatic cording	0	83	71
MLN/ICLN enlarged	0	80	50

* Results (except mean condition score) are expressed as the percentage of each group showing signs.
MLN/ICLN, mesenteric and ileocaecal lymph nodes.

mucosal ridges and crevices were often noted (in 43% and 26% of cases, respectively). Small-intestinal contents were usually normal in appearance. The ileal serosa was usually oedematous with a slightly granular appearance and with prominent cording of the serosal lymphatic ducts (83% of cases). Mesenteric and ileocaecal lymph nodes (MLN and ICLN) were enlarged in 80% of cases, usually oedematous, and occasionally showed cortical yellow-orange pigmentation. Gross lesions were always most striking at the terminal ileum adjacent to the ileocaecal valve, and became less severe more proximally and distally (Table 2). In multibacillary animals, lesions always extended proximally to the mid-jejunum, sometimes to the proximal jejunum and occasionally into the duodenum (3% of cases). A segmented distribution pattern of lesions within the ileum and jejunum was noted in only 3% of cases. Large intestinal involvement with caecal (74% of cases) and colonic (47% of cases) lesions was common, but rectal lesions were not visible macroscopically. Sheep with soft faeces or diarrhoea always had large intestinal lesions, usually extending to the colon. In 6% of multibacillary sheep, diffusely distributed, multifocal, small, pale lesions were observed in the liver. Gross lesions of paratuberculosis were not detected in any other organ, but it should be noted that one multibacillary sheep with a purulent pneumonia was excluded from the study.

Gross lesions of the ileum, including wall thickening and mucosal granularity and reddening, were obvious in 85% of the paucibacillary sheep but usually less marked than in multibacillary animals. In 15% of cases no gross intestinal lesions were apparent, despite severe clinical disease and histological lesions. No sheep in the paucibacillary group showed mucosal pigmentation, but

Table 2
Gross and histological lesion distribution in diseased sheep

Site	*Results* in*			
	multibacillary sheep (n = 31)		*paucibacillary sheep (n = 14)*	
	Gross lesions	*Histological lesions*	*Gross lesions*	*Histological lesions*
Duodenum	3	3	0	9
Jejunum	100	100	68	68
Ileum	100	100	85	100
Caecum	74	100	46	80
Colon	47	79	15	33
Rectum	0	25	0	0
MLN/ICLN	80	97	50	93
Liver	6	61	0	38
Lung	0	3	0	0

* Results are given as the percentage of each group found to have lesions.
MLN/ICLN, mesenteric and ileocaecal lymph nodes.

mucosal ridges (31% of cases) and crevices (8% of cases) were recognized; serosal lymphatic cording and regional lymph node enlargement were less apparent and present in fewer animals (71% and 50%, respectively) than in the multibacillary group. Gross lesions of the gut were less extensive in paucibacillary sheep than in multibacillary sheep; in 68% of cases they extended proximally into the jejunum, and in 46% and 15% distally into the caecum and colon, respectively. Lesions of paratuberculosis were not noted in the duodenum or rectum, or in other organ systems.

Caseation necrosis with calcification was not observed macroscopically in any animal. In a few animals (6% multibacillary and 7% paucibacillary) small focal areas of calcification occurred in the mesenteric and ileocaecal lymph nodes. Sheep were found to be pregnant in 10% of multibacillary and 21% of paucibacillary cases. Control sheep were free of significant lesions and none was pregnant.

Histopathology

Histological grading of lesions in sections of ileum from diseased sheep is summarized in Table 3. Intestine and regional lymph nodes from the control sheep showed no cellular infiltration (grade 0) or acid-fast bacilli.

Multibacillary animals. The ileum of multibacillary animals was, in 94% of cases, infiltrated by sheets of closely packed macrophages with abundant, weakly eosinophilic cytoplasm. This infiltrate distended the lamina propria and often spread into the submucosa (Fig. 1). In the remaining 6% of cases, macrophages formed near-coalescing multifocal granulomatous aggregates in the mucosa. Macrophage borders were usually reasonably distinct but "epithelioid" sheets were apparent in some areas. Multinucleate Langhan's

Table 3
Histopathological findings in diseased sheep

Observation	*Results* in each stated grade (0–3) in*							
	31 multibacillary sheep				*14 paucibacillary sheep*			
	0	*1*	*2*	*3*	*0*	*1*	*2*	*3*
Mucosa								
Lymphocytes	3	32	65	0	0	7	57	36
Macrophages	0	0	29	71	0	71	29	0
Neutrophils	48	48	4	0	29	64	7	0
Eosinophils	90	10	0	0	71	21	8	0
Giant cells	90	7	3	0	57	14	29	0
Acid-fast %	0	0	3	97	57	29	14	0
Acid-fast numbers	0	0	6	94	57	43	0	0
Submucosa								
Lymphocytes	3	78	16	3	0	50	43	7
Macrophages	6	52	16	26	14	86	0	0
Neutrophils	55	42	3	0	64	36	0	0
Eosinophils	100	0	0	0	86	14	0	0
Giant cells	97	3	0	0	86	14	0	0
Acid-fast %	6	16	13	65	79	21	0	0
Acid-fast numbers	6	16	36	42	86	14	0	0
Serosa								
Lymphocytes	19	81	0	0	29	71	0	0
Macrophages	61	19	13	7	64	29	7	0
MLN/ICLN								
Granulomas	3	22	53	22	7	43	29	21
Acid-fast numbers	40	33	20	7	79	14	0	7

* All figures are expressed as a percentage of sheep in each group (multibacillary or paucibacillary). Grading denotes degree of infiltration in the case of cell type (0, normal; 3, marked infiltration/presence). "Acid-fast %" refers to the percentage of macrophages infected (0, none; 1, <20%; 2, 20–75%; 3, >75%). "Acid-fast numbers" refers to the approximate number of bacteria seen in each macrophage (0, none; 1, <10; 2, 10–60; 3, >60). MLN/ICLN, mesenteric and ileocaecal lymph nodes. Macrophage distribution (D, F or S—see Materials and Methods) was as follows: multibacillary sheep—D 94%, F 6%, S 0% (in mucosa), and D 41%, F 45%, S 14% (in submucosa); paucibacillary sheep—D 21%, F 71%, S 8% (in mucosa), and D 0%, F 68%, S 31% (in submucosa).

giant cells were noted in a minority of cases (10%). Lymphocytic infiltration into the lamina propria was significant in 97% of cases, but usually mild to moderate and the cells were diffusely distributed amongst the macrophages. Neutrophil polymorph infiltration, which was both diffuse and focal but usually mild, occurred in 52% of cases. The cellular infiltrate surrounded and compressed the crypt epithelial glands. Crypt abscesses and local necrosis were evident in places. There was also marked villous atrophy and fusion, resulting in a substantially flattened mucosal surface (Fig. 2). Similar but less dense infiltrates extended through the muscularis layer into the submucosa in the majority of cases (94%). Macrophage sheets were more disaggregated here and usually present as multifocal granulomata. The submucosa often showed lymphatic dilation and oedema and most lymphocytes had a perivascular or perilymphatic distribution (Fig. 3). Muscle band layers appeared hypertrophied but lacked infiltration of inflammatory cells. In 81% of cases the serosa was

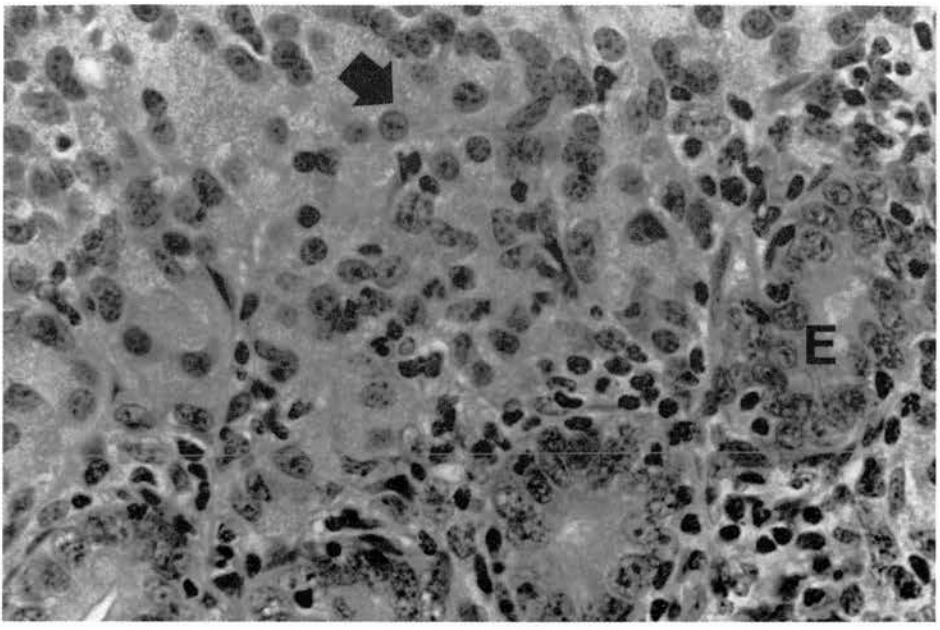

Fig. 1. Multibacillary case. Typical lesion in the intestinal mucosa showing marked infiltration into the lamina propria by macrophages with abundant cytoplasm (arrow). Surviving crypt epithelial glands (E) are pushed apart. HE. ×40.

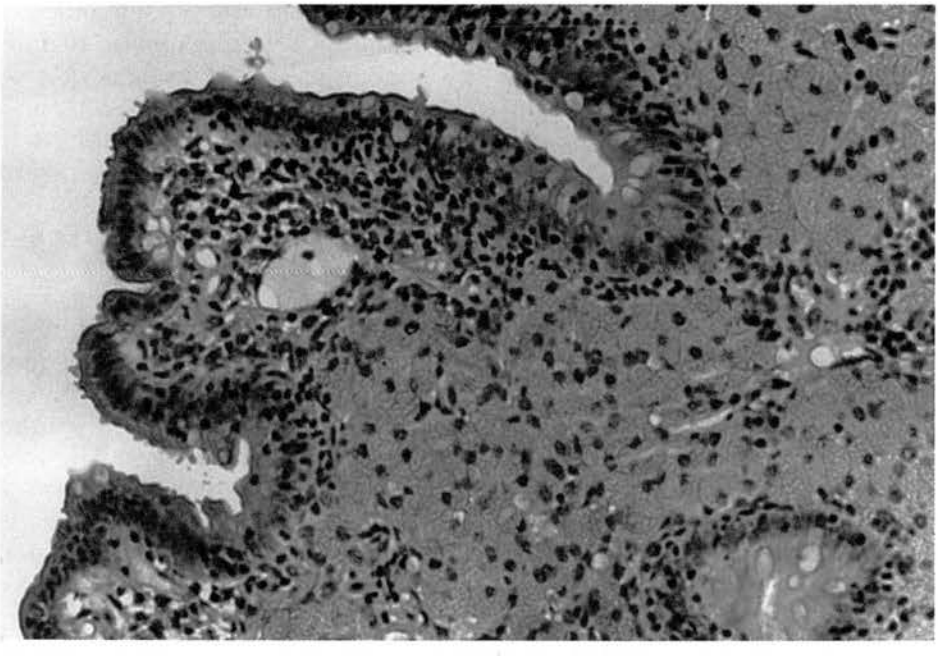

Fig. 2. Multibacillary case. Atrophic intestinal villi showing flattening and fusion, with marked macrophage infiltration beneath. HE. ×20.

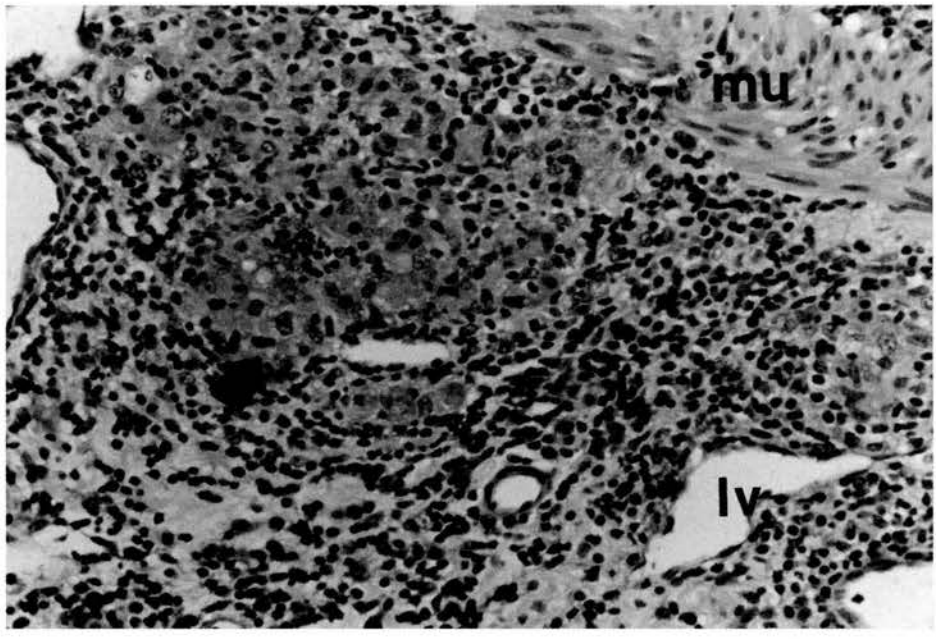

Fig. 3. Multibacillary case. Infiltration beneath the intestinal muscularis layer (mu) of the submucosa by inflammatory cells. Macrophages forming a loose granuloma (arrow) and lymphocytes (L) are evident. Lymphatic vessels (lv) are dilated. HE. × 20.

oedematous, with lymphatic dilation and a mild infiltration of lymphocytes, distributed both diffusely and focally, particularly in perilymphatic regions. Lymphangitis with a granulomatous component was noted in 32% of cases (Fig. 4).

In 97% of multibacillary cases, the mesenteric and ileocaecal lymph nodes had multifocal small granulomata composed of aggregates of large macrophages distributed throughout the cortex (Fig. 5). Occasionally the granulomata formed larger coalescing lesions. Large macrophages were also noted in the subcapsular sinus and medullary sinuses. Lymphoid hyperplasia was usually evident.

Ziehl-Neelsen staining of sections of ileum invariably demonstrated, in the lamina propria, large numbers of acid-fast rods in the cytoplasm of most macrophages and giant cells, when present (Fig. 6). In the submucosa, macrophages were less frequently infected and the numbers of intracellular organisms were small; only a few bacteria were noted in serosal macrophages. The acid-fast load was also less in the regional lymph nodes than in the nearby mucosa. Sheep with pigmented lesions always showed many acid-fast organisms and marked macrophage infiltration. However, multibacillary sheep with non-pigmented lesions had histological lesions and acid-fast loads equivalent to those of sheep demonstrating pigmentation.

Paucibacillary animals. Tissues from the paucibacillary group showed a different histological pattern, although the general signs of villous flattening, mucosal

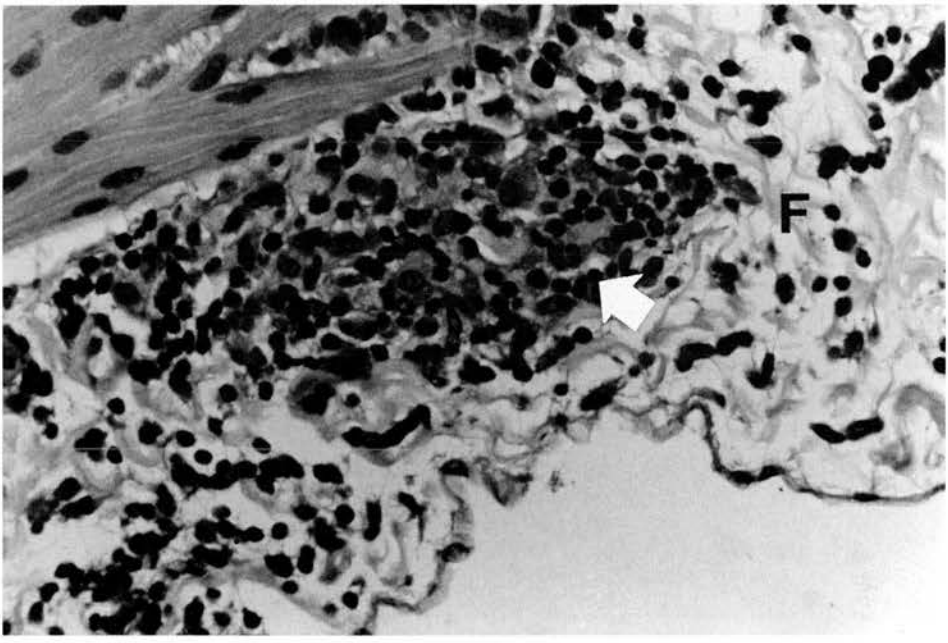

Fig. 4. Multibacillary case. Serositis with macrophages and lymphocytes in a loose granuloma (arrow) and oedema fluid (F). HE. ×40.

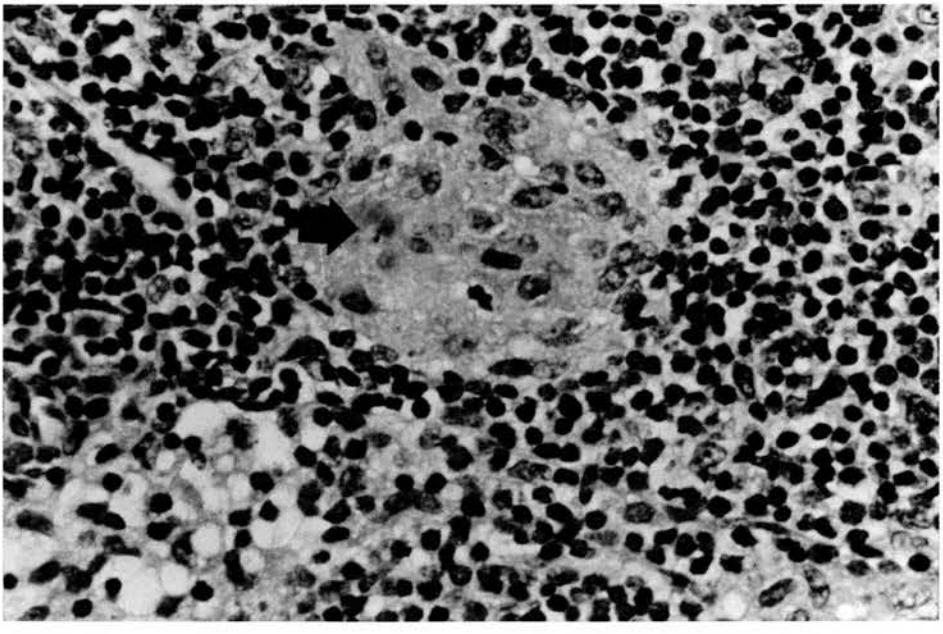

Fig. 5. Multibacillary case. Granuloma (arrow) in the mesenteric lymph node cortex. HE. ×40.

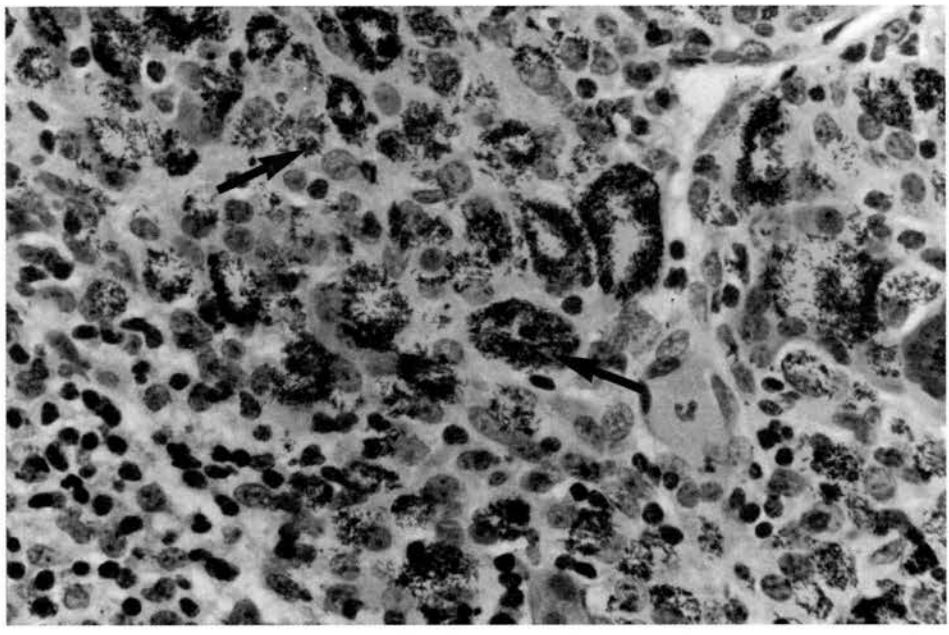

Fig. 6. Multibacillary case. Numerous acid fast rods (arrows) pack the cytoplasm of macrophages infiltrating the intestinal lamina propria. ZN. ×40.

and submucosal inflammation and oedema were similar to those of the multibacillary lesions (Table 3) (Fig. 7). The ileal lamina propria was infiltrated predominantly by sheets of small lymphocytes in 93% of cases. Large macrophages were always present too, but usually as multifocal small granulomata (71% of cases) or isolated groups of a few cells (8% of cases) surrounded by the lymphocytic infiltrate (Fig. 8). These small granulomata were never encapsulated and showed no evidence of necrosis or calcification. More diffuse macrophage infiltrates, seen in 21% of cases, were usually not as extensive and were associated with a greater lymphocyte presence than in the multibacillary group. Langhan's giant cells, often arranged together, were evident in 43% of cases (Fig. 9). Mild infiltration by granulocytes was noted in 64% of cases. Lesions extended into the submucosa and continued to reflect the predominance of lymphocytes, all macrophages being distributed in scattered small groups or singly. Giant cells were noted in the submucosa in 14% of cases. Serosal changes were similar to those seen in the multibacillary group, although the oedema was usually less. Small granulomata were noted in the cortices of the associated regional lymph nodes in 93% of paucibacillary cases.

Ziehl-Neelsen staining of the paucibacillary tissues indicated far fewer acid-fast organisms than in the multibacillary group (Fig. 10). Careful scanning of sections of ileal mucosa and lymph nodes revealed acid-fast bacteria in the macrophage cytoplasm in only 50% of cases. In these animals, the organisms were generally present in fewer than 75% of cells and in numbers of <10/ cell section. The acid-fast load was even lower in the submucosal and regional

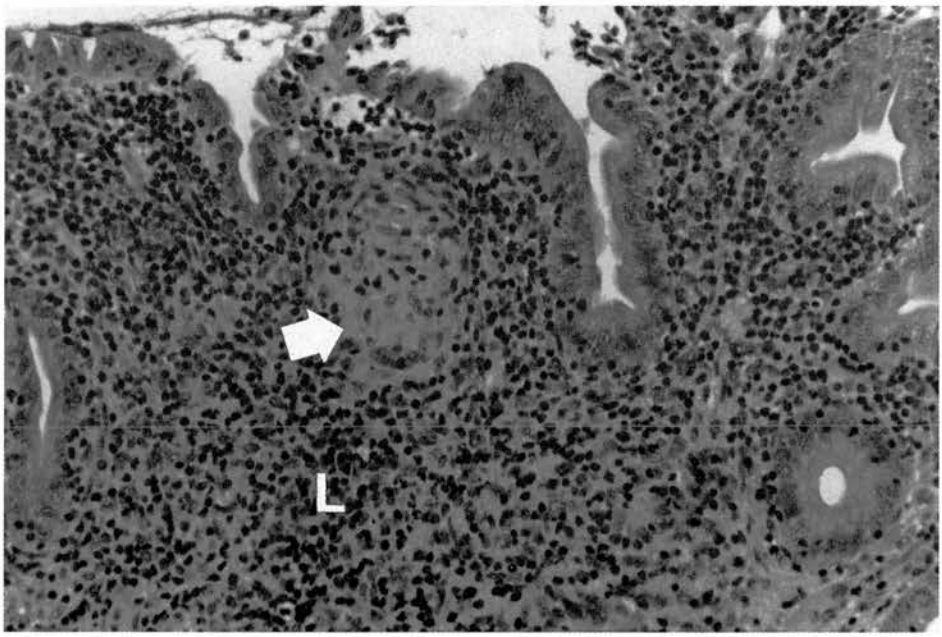

Fig. 7. Paucibacillary case. Villous atrophy associated with a marked lymphocytic infiltrate (L) into the intestinal mucosa. A focal granuloma is evident (arrow). HE. ×20.

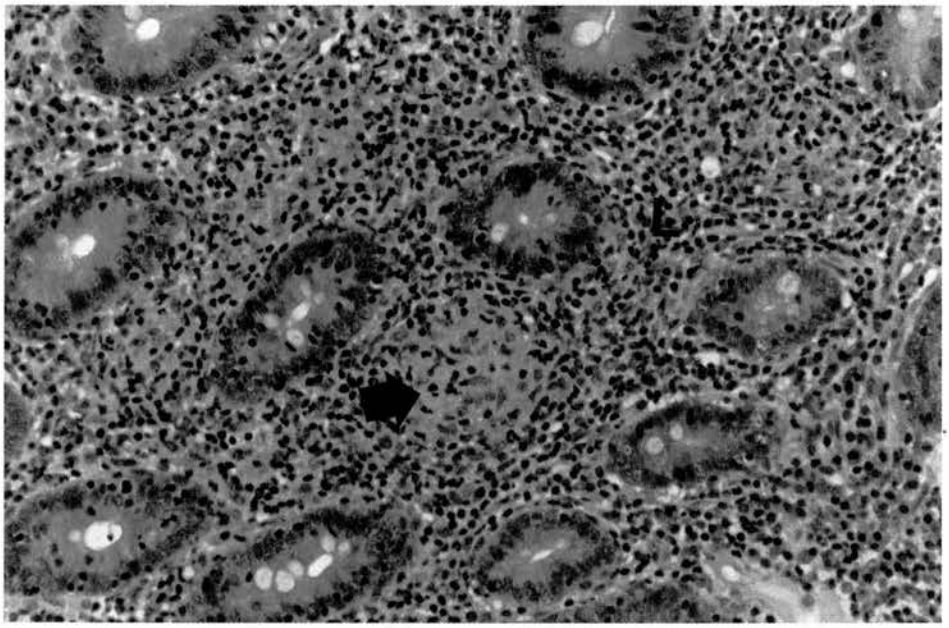

Fig. 8. Paucibacillary case. Mucosal lamina propria infiltrate showing focal granuloma (arrow) and lymphocytes (L) between crypt epithelial glands. HE. ×20.

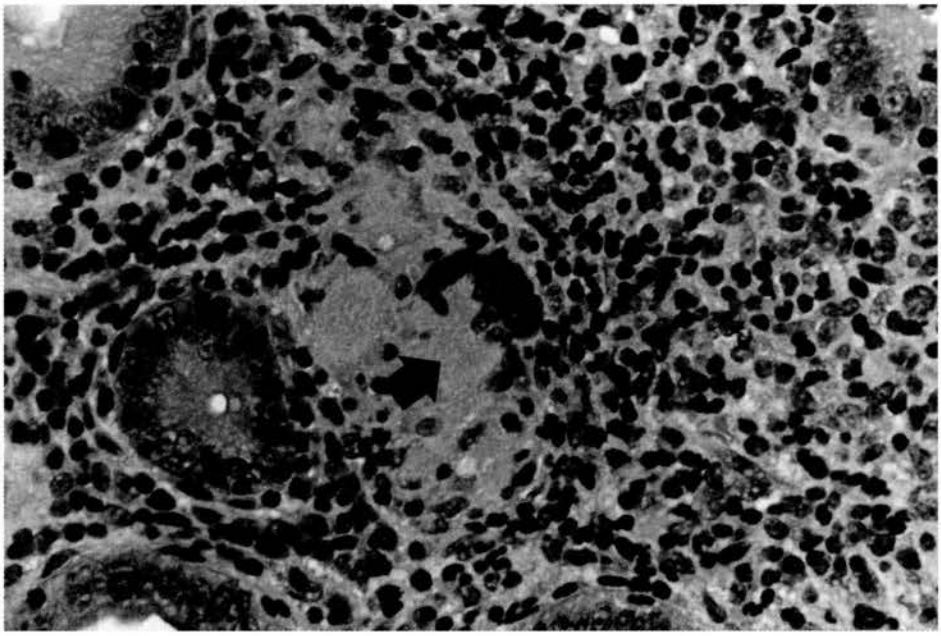

Fig. 9. Paucibacillary case. Multinucleate giant cell (arrow) and macrophages in the intestinal mucosa. HE. ×20.

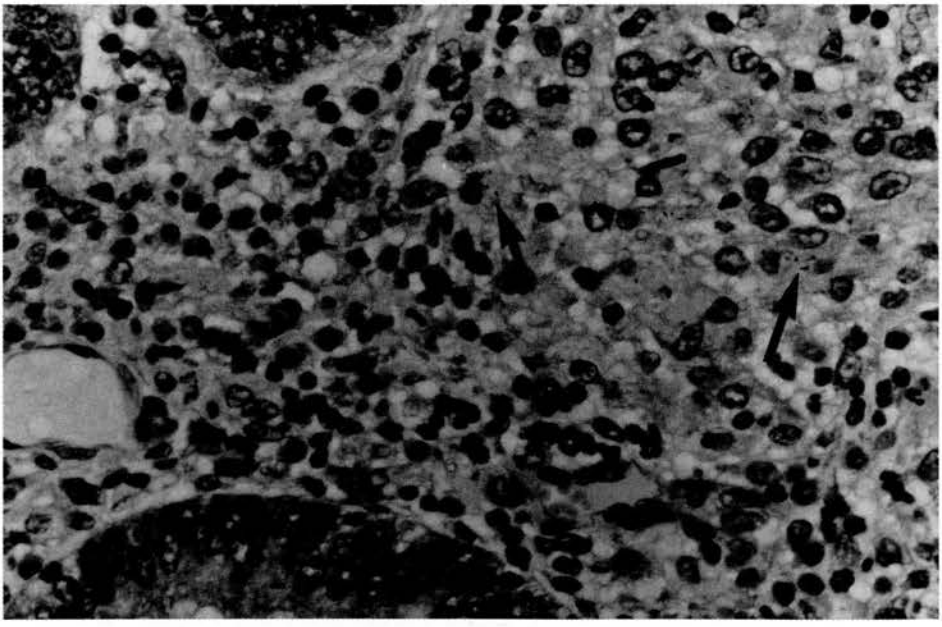

Fig. 10. Paucibacillary case. Intestinal granuloma with very few acid-fast rods (arrows) in the cytoplasm of macrophages. ZN. ×40.

lymphoid tissue, with only 21% of cases having organisms in these regions. No animal classified as paucibacillary had pigmented gut lesions.

Toluidine blue staining for mast cells revealed only a very modest, insignificant increase in this cell type in diseased ileum compared with controls (data not shown). Examination of calcified lesions in mesenteric and ileocaecal lymph nodes from the few affected multibacillary and paucibacillary sheep showed no convincing evidence that they were associated with mycobacterial granulomata.

Statistical Analysis

Analysis of these results from both groups of diseased sheep showed a highly significant correlation ($P<0{\cdot}001$) between macrophage infiltration into the ileal mucosa and both the percentage of macrophages containing acid-fast organisms and the mean organism load per cell. The correlation between the proportion of cells infected with acid-fast bacteria and the bacterial load per cell was also strong ($P<0{\cdot}001$). There was an inverse correlation between lymphocyte infiltration and acid-fast presence ($P<0{\cdot}001$). Lymphocyte infiltration and macrophage infiltration also had a strong inverse correlation ($P<0{\cdot}001$). The presence of giant cells was significantly correlated with lymphocyte infiltrate ($P<0{\cdot}02$) and inversely correlated with bacteria ($P<0{\cdot}01$ for both percentage of cells infected and mean organism load per cell).

A significant correlation was found between the histological presence of bacteria in the gut and a positive serum AGID test ($P<0{\cdot}01$).

Tissues other than Ileum and Regional Lymph Nodes

Other regions of the intestine were found to have lesions similar to those found in the ileum, but they became less pronounced, with less submucosal and serosal involvement and fewer acid-fast bacteria, as distance from the ileum increased (Table 2). Histological examination was a more sensitive method of lesion detection than macroscopical examination. The ileum and regional lymph nodes were the most likely sites of lesions. The majority of sheep in both bacillary groups had histological lesions in the ileum, jejunum and caecum, but colonic and duodenal lesions occurred less frequently and rectal lesions were undetectable in 75% of multibacillary and 100% of paucibacillary cases (Fig. 11).

Granulomatous lesions were also found in the liver (61% of multibacillary cases and 38% of paucibacillary cases) and lung (3% of multibacillary cases), but acid-fast organisms were either not detected or present in only small numbers in these sites. Lesions were not seen in peripheral lymph nodes, spleen, kidney, heart, mammary gland, uterus or fetal tissue. Liver lesions usually consisted of multifocal small granulomata scattered throughout the parenchyma and occasionally distributed periportally (Fig. 12). Lung lesions had a peribronchial distribution pattern.

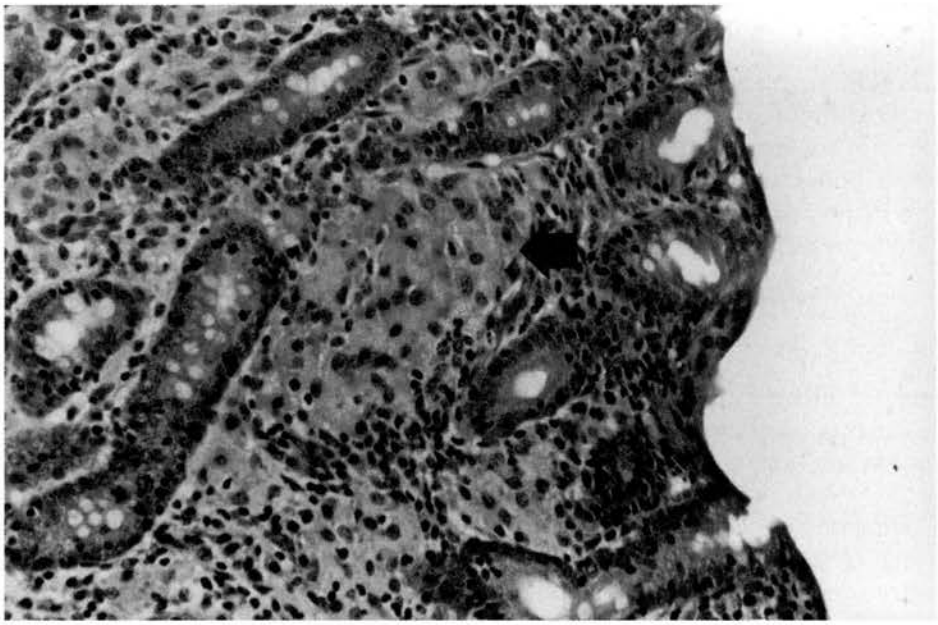

Fig. 11. Multibacillary case. Colonic mucosa showing infiltration by macrophages (arrow). HE. ×20.

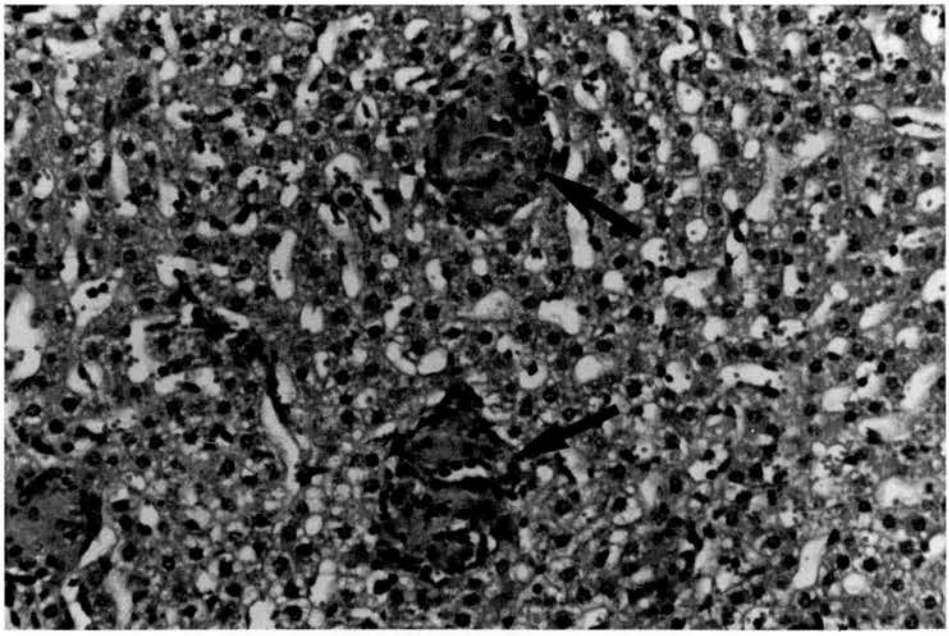

Fig. 12. Multibacillary case. Liver parenchyma with focal granulomas (arrows). HE. ×20.

Polymerase Chain Reaction (PCR)

All the ileal tissues tested from multibacillary sheep gave a positive PCR result for specific *M. a. paratuberculosis* DNA. The paucibacillary sheep tissues were positive by PCR in nine of 14 cases. Of the remaining five cases, two showed histological evidence of acid-fast organisms in the intestine and mesenteric lymph nodes, a further two gave a positive AGID test result, and the remaining animal gave a strong interferon-γ response when blood lymphocytes were stimulated by *M. a. paratuberculosis* antigen in an in-vitro assay (Rothel *et al.*, 1990) (performed by Dr C. Burrells, Moredun Research Institute, Edinburgh). All control sheep tissues tested gave a negative result with the PCR test.

Discussion

This study revealed two distinct histological types of granulomatous enteritis in ovine clinical paratuberculosis and demonstrated significant relationships between the infiltrating cell type and the degree of intestinal mycobacterial infection. The majority of the diseased sheep (69%) belonged to the so-called multibacillary group; these animals had an extensive, diffuse macrophage infiltrate within the intestinal mucosa and submucosa. The so-called paucibacillary group (31% of cases) showed a marked lymphocytic and sometimes giant cell infiltration into the intestine. Macrophages in the paucibacillary group were present in small granulomata. Macrophage infiltration was significantly correlated with the presence of acid-fast bacteria. Lymphocytic and macrophage infiltrations were inversely correlated with each other, suggesting the occurrence of antagonistic pathological processes.

The multibacillary group corresponded substantially to the previously described ovine paratuberculosis pathology type I of Stamp and Watt (1954), group I of Rajya and Singh (1961), type I of Reddy *et al.* (1984), the majority of cases described by Carrigan and Seaman (1990) and type IIIb of Perez *et al.* (1992). The paucibacillary group corresponded to similar cases described by Stamp and Watt (1954) (group II), Rajya and Singh (1961) (group II), Carrigan and Seaman (1990) (the minority of cases) and Perez *et al.* (1992) (group IIIc). Giant cells were often seen in these cases but, in contrast to the findings of others (Stamp and Watt, 1954; Carrigan and Seaman, 1990; Perez *et al.*, 1992), they were also found in a number of our multibacillary cases. The multibacillary form of paratuberculosis has histological similarities with lepromatous leprosy, macrophage infiltrates sometimes forming "epithelioid cell" sheets with a mosaic pattern. The lesions of the paucibacillary form seem comparable with tuberculoid and borderline lepromatous lesions (Ridley, 1974).

Histological grouping into multibacillary and paucibacillary types made possible a retrospective examination of possible correlations with other data, including clinical and serological findings and gross lesions. Most clinical signs and gross lesions were of limited use in indicating the type of pathology present, except in those multibacillary cases in which pigmented mycobacteria caused obvious pigmentation in the gut. No significant difference in histological

lesions was found between pigmented and non-pigmented multibacillary cases, suggesting that the causative strains were pathogenically similar. Virtually all diseased sheep in this study were in poor condition and had intestinal thickening and lymphangitis. Inflammatory cell infiltrates were responsible for much of the increased thickness in the mucosa and submucosa and for the villous atrophy. In individual animals, lesions throughout the gut always tended to reflect the same type of pathology. The lesions were most marked in the terminal ileum, the apparent site of primary infection (Momotani *et al.*, 1988). In affected sheep, in contrast to cattle, diarrhoea was not a constant feature, being present in only 19% of multibacillary cases. Diarrhoea and soft faeces occurred mainly in those cases with large intestinal (particularly colonic) lesions, probably as a result of interference with fluid reabsorption.

Mycobacteria were always most numerous in the intestinal mucosa, less numerous in the submucosa and regional lymph nodes, and usually absent or sparse in other organs. This reflects the localization of lesions in paratuberculosis and suggests either a particular susceptibility of the intestine to infection or a more robust immune response in other tissues, or both.

In contrast to other studies (Stamp and Watt, 1954; Rajya and Singh, 1961) the present study did not demonstrate tuberculous type lesions with caseation necrosis or calcification. The few calcified foci that were detected showed no evidence of being lesions of paratuberculosis and were tentatively attributed to previous parasitic infections. Early type focal granulomatous lesions of lymphoid tissues, as described in young sheep by Nisbet *et al.* (1962) and Perez *et al.* (1992), were also not found, probably because the animals were adult sheep with advanced clinical disease.

Positive serum AGID test results were significantly correlated with the presence of acid-fast bacteria in the gut, showing that a substantial but evidently ineffective humoral response was elicited in most multibacillary cases. This may reflect immune recognition of the mycobacteria as they are released when infected macrophages die and disintegrate (Chiodini *et al.*, 1984). In contrast, the very low mycobacterial load of paucibacillary cases would seem to be associated with poor humoral immunity. However, the substantial lymphocyte recruitment into the gut of paucibacillary cases would seem consistent with a cell-mediated immune response. We did not investigate whether the lymphocytic infiltration was caused by the low acid-fast presence, nor did we investigate the function of the lymphocytes. In other mycobacterial diseases, various antibody-helper, delayed-type hypersensitivity, cytotoxic or even suppressor roles have been suggested for infiltrating lymphocytes (Modlin *et al.*, 1986; Arnoldi *et al.*, 1990; Flynn *et al.*, 1992; Orme *et al.*, 1993). The presence of severe intestinal lesions with few or no mycobacteria suggests an immunopathological mechanism.

These findings have implications for the diagnosis of ovine paratuberculosis. In multibacillary animals, diagnosis was assisted by the serum AGID test, the heavy presence of intestinal mycobacteria as reflected in faecal examination and culture, and histological examination of the ileum and regional lymph nodes. Rectal biopsy seemed less likely to be useful, as only 25% of cases had rectal lesions. Diagnosis of the paucibacillary cases was more difficult because

the AGID test was usually negative and there was little likelihood of detecting acid-fast organisms in faeces. Histological examination of the ileum and of the lymph nodes were both highly sensitive diagnostic procedures (100% and 93% sensitivity, respectively), but no case had detectable rectal lesions. Mycobacterial culture from faeces is always a prolonged process and is often unsuccessful with sheep strains (Carrigan and Seaman, 1990; Collins *et al.*, 1993; Jubb *et al.*, 1993). In our study, intestinal tissues were subjected to PCR analysis for specific *M. a. paratuberculosis* DNA. All multibacillary sheep gave a positive result, but a minority of paucibacillary sheep were negative, possibly because of insufficient test sensitivity or a genuine absence of mycobacteria in the sample. However, strong evidence of paratuberculosis in these PCR-negative animals was provided by a positive AGID test (regarded as completely specific; Hilbink *et al.*, 1994), or the presence of acid-fast bacilli in lesions, or a positive interferon-γ blood lymphocyte stimulation test. All these animals also had clinical, macroscopical and histological features resembling those of the other paucibacillary sheep. Acid-fast organisms were not detected histologically in 50% of paucibacillary cases. Similar observations have been made previously (Stamp and Watt, 1954; Jubb *et al.*, 1993). In our paucibacillary cases, the intestinal and lymphoid lesions were the same, regardless of the presence or absence of detectable acid-fast rods. Furthermore, sheep with no detectable mycobacteria gave either a positive serum AGID test, PCR result or interferon-γ test result.

The two pathological types of ovine paratuberculosis may represent different stages of the same process. Alternatively, they may be the result of different pathogenic mechanisms, probably related to the host immune response. A few animals in this study had lesions of an intermediate type, which may have reflected a transitional type of disease. Furthermore, unlike Perez *et al.* (1992), we found that the correlation of low acid-fast presence with a negative AGID test was not absolute, a number of paucibacillary sheep giving a positive AGID test result. This may have been due to persistent antibody from a previous, more multibacillary infection. Intermediate types of pathology, depending on immune status and other factors, are recognized in other mycobacterioses such as leprosy (Ridley, 1974).

In conclusion, the two pathological types of ovine paratuberculosis may have similar clinical and gross pathological features, but they differ in (1) type of cellular infiltrate, (2) degree of mycobacterial colonization, and (3) serum antibody response. It is possible that the pathogenesis of paratuberculosis depends in multibacillary sheep on a largely failed immune response to the mycobacteria, and in paucibacillary sheep on an inappropriate cell-mediated immune response. The mechanisms that lie behind the development of the two pathological types require further investigation.

Acknowledgments

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Phenotypic characterisation of intestinal lymphocytes in ovine paratuberculosis by immunohistochemistry

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Abstract

Characterisation of the T-cell subsets in intestinal lesions in sheep with paratuberculosis may contribute to our understanding of the pathogenesis of this disease.

To determine the phenotype and distribution of lymphocytes in the normal sheep intestinal mucosa and in *Mycobacterium avium* subspecies *paratuberculosis* infected sheep, immunohistochemistry was performed on 12 normal sheep and 18 naturally infected, clinically diseased sheep of which 12 showed lepromatous and six tuberculoid forms of the disease. Immunoperoxidase staining was carried out on frozen sections of ileum using monoclonal antibodies against ovine CD4, CD8, and $\gamma\delta$ ·T-cell receptor (TCR) markers.

In all three sample groups, cells appeared to be non-randomly distributed throughout the lamina propria. Higher densities of lymphocytes were present in villus than in crypt areas. $CD8^+$ cells were located principally around the epithelial basement membrane, whereas $CD4^+$ cells were localised towards the central villus area of the lamina propria. Lymphocytes bearing the $\gamma\delta$ T-cell receptor were more widely distributed, both in epithelial and lamina propria compartments. Ileum with tuberculoid lesions had higher densities of CD4 and $\gamma\delta$ T-cell subsets while lepromatous lesions had lower densities of CD4 and CD8 cells compared with normal tissues. The median relative percentage of $CD4^+$ cells was increased and that of $CD8^+$ cells decreased in tuberculoid cases, with a corresponding increase in the CD4:CD8 ratio, while the relative percentage of $\gamma\delta^+$ cells was increased in lepromatous cases.

Keywords: *Mycobacterium avium* subspecies *paratuberculosis*; Intestinal lymphocytes; Sheep; Immunohistochemistry

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1. Introduction

Paratuberculosis (Johne's disease) is an intestinal mycobacteriosis of ruminants which is manifest as a chronic granulomatous enteritis, and is caused by *Mycobacterium avium* subspecies *paratuberculosis* infection (Chiodini et al., 1984; Thorel et al., 1990). The immunology of paratuberculosis in sheep has not been extensively documented. Most immunological studies in paratuberculosis have been performed in the bovine, in particular with reference to peripheral blood lymphocytes and their responses to mycobacterial antigens (Kreeger and Snider, 1992; Kreeger et al., 1992; Chiodini and Davis, 1992; Chiodini and Davis, 1993). This study is concerned with the lymphocytes of the distal ileum, which is the consistent site of infection and lesions in the sheep.

A spectrum of lesions is seen in ovine paratuberculosis, varying from small focal granulomatous areas with a predominantly lymphoid cell infiltrate, low numbers of infected macrophages with low intracellular bacterial burdens, with or without the presence of giant cells (tuberculoid type), to diffuse, extensively affected areas containing high numbers of large macrophages with heavy intracellular bacterial burdens and relatively few lymphocytes (lepromatous type) (Stamp and Watt, 1954; Rajya and Singh, 1961; Reddy et al., 1984; Carrigan and Seaman, 1990; Pérez et al., 1996; Clarke and Little, 1996).

In paratuberculosis, the persistence of the infected macrophage suggests an apparent failure of the host's local enteric immune response to eliminate the pathogen by cell-mediated immune mechanisms normally employed against intracellular pathogens.

Lymphocyte phenotype has been linked to important immune functions in a variety of mycobacterial infections. In the mouse, $CD4^+$ cells have been categorised on the basis of function into Th1 and Th2 subpopulations (Mosmann et al., 1986). In an effective immune response $CD4^+$ helper T-cells of Th1 type produce gamma interferon (IFNγ) which promotes intracellular killing by infected macrophages (Nathan et al., 1983). A Th2 type of response resulting in downregulation of Th1 type responses and the promotion of antibody production would appear to be inappropriate in a mycobacterial infection. $CD8^+$ cells are necessary for a protective immune response to *M. tuberculosis* infection (Flynn et al., 1992), and are present in large numbers in lepromatous leprosy lesions in which a suppressor role has been suggested (Modlin et al., 1986). Gamma-delta T cells are a prominent lymphocyte subset in ruminants (Hein and Mackay, 1991), accumulate at the site of mycobacterial infections (Janis et al., 1989; Modlin et al., 1989) and are considered to be involved in the regulation of granuloma formation (Mombaerts et al., 1993). Investigation of the T-lymphocyte subsets present in normal and paratuberculosis infected ileum may provide information on the persistence of infection.

In this study, we have attempted to characterise phenotypically the T-cell subsets of the ileum in situ by immunohistochemistry. The proportions and distribution of intestinal lymphocyte subsets have been examined both in normal, non-infected sheep, and in sheep with clinical paratuberculosis.

2. Materials and methods

2.1. Animals

Thirty adult sheep of Scottish Blackface and Cheviot breeds, comprising 18 adult naturally paratuberculosis infected, clinically diseased sheep and 12 healthy adult sheep were euthenised by intravenous barbiturate injection. Sheep were given a clinical examination ante-mortem, and serologically tested for *M. a. paratuberculosis* antibodies using the agar gel immunodiffusion (AGID) test (Sherman et al., 1984).

2.2. Samples

Full necropsy examinations were carried out during which fresh samples of distal ileum were collected and snap frozen in solid CO_2/isopentane slush and stored in OCT cryoprotectant compound (Miles, Elkhart, IL, USA) at −70°C until required. Gut samples were also taken and fixed in buffered formalin solution and processed routinely for histological examination. Sections were cut and stained with haematoxylin and eosin, and by the Ziehl-Neelsen method for the detection of acid-fast organisms.

2.3. Immunohistochemistry

Serial sections of frozen ileum were cut at 6–8 microns on a cryotome onto glass slides which had been pre-treated with Vectabond tissue section adhesive (Vector Labs. Peterborough, UK) and allowed to air dry at room temperature for 2 h. Sections were fixed in cold acetone at 4°C for 5 min and allowed to air dry before being mounted in coverplates (Shandon, Basingstoke, UK), and washed in phosphate buffered saline, pH 7.4. Endogenous peroxidase was blocked by incubating with glucose oxidase (Andrew and Jasani, 1987) for 1 h at 37°C. Primary monoclonal antibodies were added to the sections and incubated overnight (approximately 16 h) at 4°C. Monoclonal antibodies used were 'SBUT4' and 'SBUT8' against ovine CD4 and CD8 leukocyte antigens respectively (Maddox et al., 1985), and '86D' against ovine $\gamma\delta$ T-cell receptor (Mackay et al., 1989). Normal mouse serum was used as a negative control. Sections were stained using the VectorStain Elite ABC streptavidin/biotin immunoperoxidase kit (Vector). Positive staining was visualised using diaminobenzidine (DAB) (Vector) as the chromogen, and the product darkened by the addition of nickel chloride solution to the reaction. Sections were counterstained in Meyer's haematoxylin, cleared through graded ethanol and xylene, and mounted in DPX mountant (BDH, Poole, UK).

2.4. Counting

Positively stained cells were enumerated through a microscope at 200× magnification using a rectangular graticule which measured a field of 0.32 × 0.21 mm (0.0672 mm^2). For each tissue section, five graticule fields were chosen from villus areas and five from crypt areas. Within these fields, cells were counted and categorised as being epithelial-associated, or lamina proprial cells. Fields containing organised granulomas

were not included in the counts, although an assessment of T-cell subset involvement in these lesions was made.

Intraepithelial cells were quantified by counting the positively stained cells in ten lengths of epithelium, each measuring 0.32 mm, for each stained tissue section.

2.5. Statistics

'Minitab For Windows' (Minitab Inc., State College, PA, USA) was used for the statistical analysis. The Kruskall-Wallis test was applied to data for one-way analysis of variance between three groups, and the Mann-Whitney test used for analysis of difference between two groups. Statistical significance was taken to be $P < 0.05$.

3. Results

Control sheep were confirmed as being non-diseased by serology, clinical examination, necropsy and histology. Diseased sheep were found to have low body condition scores on clinical examination and some were noted to be moribund. Some sheep had evidence of diarrhoea, and some soft faeces, but this has been considered not to be a consistent finding in ovine paratuberculosis and most sheep had normal pelleted faeces. Gross and histological changes in diseased sheep have been described previously (Clarke and Little, 1996). In brief, carcases were emaciated and generally showed evidence of systemic oedema. The majority of sheep had pigmented ileal mucosae with thickening of the ileal walls and corrugation of the luminal surface. Ileal serosa was often oedematous and mesenteric lymph nodes usually notably enlarged. Paratuberculosis infection was confirmed by histopathological examination and AGID serology. Twelve animals had lepromatous (diffuse) type histopathology with the high numbers of large, infected macrophages present containing heavy burdens of acid-fast staining intracellular bacteria, and disruption of the normal ileal mucosal structure. Six animals

Table 1
Distribution of T-cell subsets within the ileal mucosa

Group	Subset	Villus total	Crypt total	LP total	EA total
Control	$CD4^+$	275.5(83–428)	77.5(42–124) **	342.5(167–498)	1(0–10) **
	$CD8^+$	372(182–726)	89.5(63–155) **	245(74–581)	241(145–465)
	$\gamma\delta^+$	62.5(43–139)	36(18–52) **	55(23–147)	41.5(18–76) *
Lepromatous	$CD4^+$	180(93–263)	69.5(32–142) **	247.5(123–349)	3(0–18) **
	$CD8^+$	225(79–356)	80(38–235) **	143.5(79–255)	153(53–277)
	$\gamma\delta^+$	66.5(34–118)	31(12–78) **	63(30–116)	27.5(10–62) **
Tuberculoid	$CD4^+$	423(361–626)	264(105–340) **	688(492–879)	2(0–10) **
	$CD8^+$	476.5(301–656)	148(116–251) **	343.5(274–461)	222(109–507)
	$\gamma\delta^+$	166.5(95–220)	67(39–98) *	120.5(85–159)	108(22–215)

LP: lamina propria. EA: epithelium associated.Data are expressed as median and range (min–max) total cell counts.Significant differences between compartments (villus and crypt, and EA and LP) are denoted by ** ($P < 0.01$) and * ($P < 0.05$).

had tuberculoid (nodular) type histopathology with a marked lymphoid cellular infiltrate and fewer infected macrophages which were generally present in focal areas, and lower intracellular acid-fast bacteria burdens.

Eleven of the 12 lepromatous, and two of the six tuberculoid animals gave a positive AGID test result.

3.1. Distribution of lymphocytes

For all animals, lepromatous, tuberculoid and non-infected, qualitative and quantitative observations showed that each T-cell subset differed in distribution throughout the mucosa (Table 1) (Fig. 1, Fig. 2, Fig. 3).

Higher densities of all three lymphocyte subsets were present in villus than in crypt areas both in infected and non-infected animals ($P < 0.01$).

$CD4^+$ cells were situated in the central lamina propria area of the villus around the central lacteal vessel, with very few $CD4^+$ cells being found within the villus and crypt epithelium ($P < 0.01$) in both infected and non-infected animals (Fig. 1a, b, c).

$CD8^+$ cells by contrast were located predominantly in close association with the epithelium around the basement membrane, and were present also as intraepithelial lymphocytes (Fig. 2a, b, c).

Gamma-delta TCR^+ lymphocytes were more widely and sporadically distributed throughout the mucosa, both in the lamina propria and within and around the epithelium, and were fewer in number than CD4 and CD8 subsets (Fig. 3 a, b, c).

In addition, the distribution of lymphocytes in association with focal granulomas was assessed, but no pattern of accumulation of T-cell subsets was apparent in these lesions.

3.2. Densities of lymphocytes

Animals showing tuberculoid type histopathology had significantly higher densities of all three cell subsets compared with animals with lepromatous type histopathology, and higher cell densities of $CD4^+$ and $\gamma\delta^+$ cells compared with control animals (Table 2).

In tuberculoid cases, there was a significantly higher density of $CD4^+$ lymphocytes than in control ($P < 0.001$) and lepromatous animals ($P < 0.001$), the $CD8^+$ density was significantly higher than lepromatous only ($P < 0.05$), and the density of the $\gamma\delta^+$ subset was higher than both lepromatous and control ($P < 0.05$).

Lower densities of both $CD4^+$ and $CD8^+$ lymphocytes were found in lepromatous than in control ileum ($P < 0.01$). However, no significant difference was found in the densities of $\gamma\delta^+$ lymphocytes between lepromatous and control ileum.

These observations were true for total ileal cells, total villus cells, and total villus lamina propria cell compartments.

For crypt areas, the densities of all three subsets were significantly higher in tuberculoid than both normal and lepromatous animals ($P < 0.05$). However, there were no significant differences between lepromatous and normal animals. This suggests that the crypt areas are more affected by the tuberculoid form of the disease than the lepromatous form.

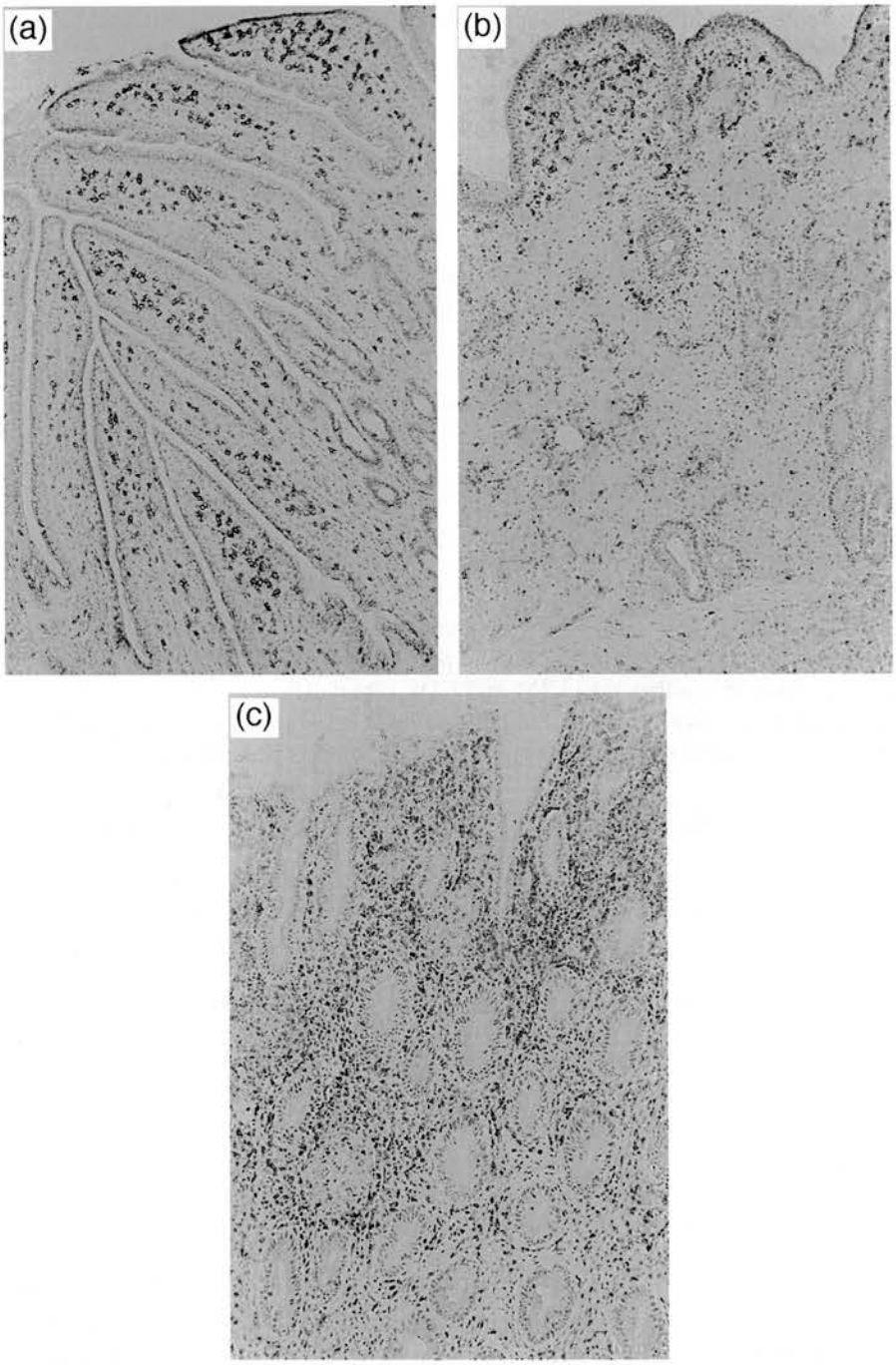

Fig. 1. Immunoperoxidase staining visualised using DAB. Medium power magnification (×10 objective). (a) Control ileum. $CD4^+$ cells. (b) Paratuberculosis infected ileum. Lepromatous type. $CD4^+$ cells. (c) Paratuberculosis infected ileum. Tuberculoid type. $CD4^+$ cells.

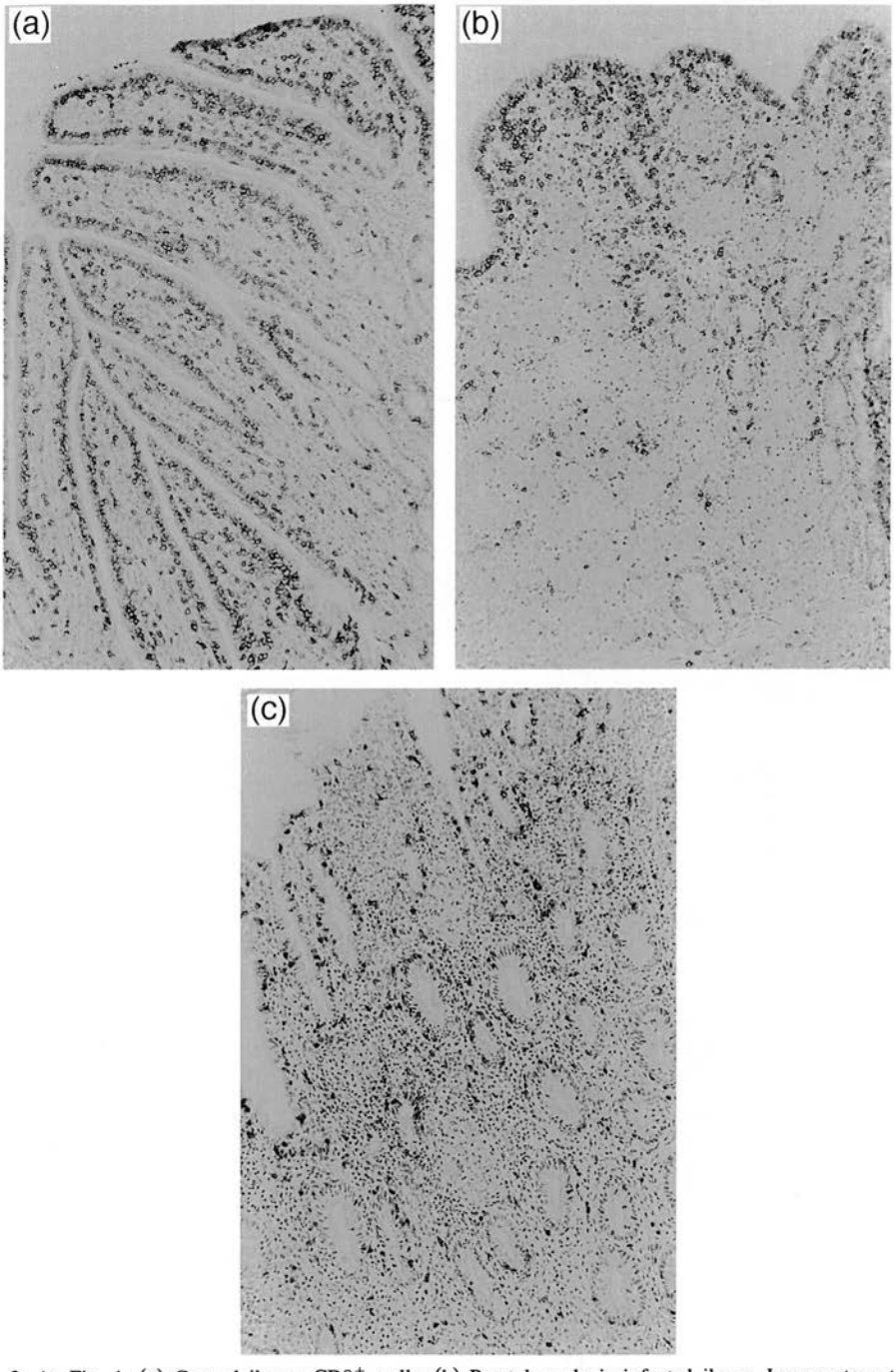

Fig. 2. As Fig. 1. (a) Control ileum. $CD8^+$ cells. (b) Paratuberculosis infected ileum. Lepromatous type. $CD8^+$ cells. (c) Paratuberculosis infected ileum. Tuberculoid type. $CD8^+$ cells.

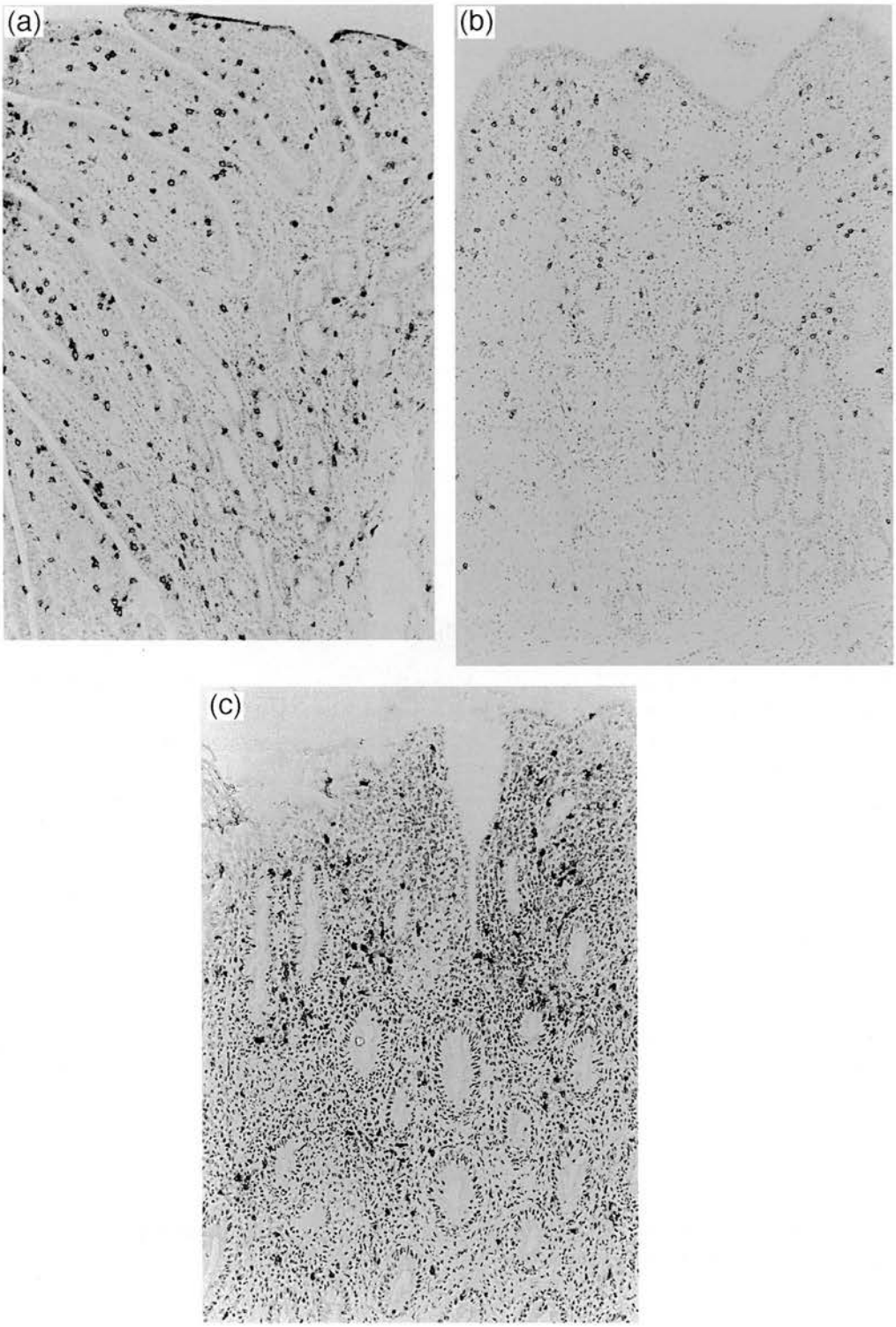
(a)
(b)
(c)

Table 2
Total cell densities, relative percentages and ratios

Observation	Subset	Control ($n = 12$)	Tuberculoid ($n = 6$)	Lepromatous ($n = 12$)
Total cells	$CD4^+$	343(167–503)	688.5(502–880) ***	250.5(125–352) $^{**\#\#\#}$
	$CD8^+$	458(255–881)	641.5(418–815)	291.5(132–532) $^{**\#\#}$
	$\gamma\delta^+$	98.5(67–187)	230(134–314) *	93(58–174) $^{\#}$
Relative %	$CD4^+$	37.0(29.2–46.8)	46.7(30.8–55.3) *	35.0(24.3–60.5)
	$CD8^+$	52.0(44.0–58.8)	40.6(30.8–50.0) **	47.5(26.8–60.4)
	$\gamma\delta^+$	9.5(8.7–16.5)	13.2(9.9–21.9)	15.0(8.8–27.8) *
Ratios:				
CD4:CD8	Total cells	0.72(0.50–1.06)	1.18(0.62–1.64) *	0.71(0.40–2.26)
	Villus cells	0.68(0.46–1.15)	1.09(0.61–1.32) *	0.70(0.36–2.22)
	Crypt cells	0.80(0.56–1.41)	1.78(0.66–2.78) *	0.88(0.56–2.32)
CD4 + CD8:$\gamma\delta$	Total cells	9.54(5.07–10.54)	6.61(3.57–9.06)	5.73(2.60–10.35) *
	Villus cells	11.93(4.90–14.27)	6.21(3.28–9.89) *	5.55(2.47–10.26) **
	Crypt cells	5.15(2.89–8.72)	6.81(2.81–11.26)	5.26(2.88–10.60)

Data are expressed as median and range (min–max).Significant differences from the control group are denoted *** ($P < 0.001$), ** ($P < 0.01$) and * ($P < 0.05$).Significant differences of tuberculoid from lepromatous group are denoted $^{\#\#\#}$ ($P < 0.001$), $^{\#\#}$ ($P < 0.01$) and $^{\#}$ ($P < 0.05$).

3.3. Relative percentages of lymphocytes

The relative percentage of each cell subset was calculated by expressing the number of cells counted for a particular subset as a fraction of all cells counted for all three subsets (Table 2).

Relative percentages of both the $CD4^+$ and $CD8^+$ subsets for lepromatous versus non-infected animals were not significantly different. However, the relative percentage of the $\gamma\delta^+$ subset was significantly greater in the lepromatous group than in the non-infected animals ($P < 0.05$).

In the tuberculoid group, the relative percentage of $CD4^+$ cells was significantly increased ($P < 0.05$), and that of $CD8^+$ cells significantly decreased ($P < 0.01$) compared with non-infected animals.

3.4. Ratios of lymphocytes

The CD4:CD8 ratios were calculated for all three groups of animals (Table 2). For control and lepromatous animals the median CD4:CD8 ratios were less than one, with $CD8^+$ cells predominating, however in tuberculoid animals the median CD4:CD8 ratio was greater than one due to a higher proportion of $CD4^+$ cells. In tuberculoid animals, the total CD4:CD8 ratio was significantly increased compared with non-infected animals ($P < 0.05$). No significant differences were found between lepromatous and control animals nor between lepromatous and tuberculoid animals.

Ratios were calculated for gamma-delta T cells versus non gamma-delta subsets.

Fig. 3. As Fig. 1. (a) Control ileum. $\gamma\delta^+$ cells. (b) Paratuberculosis infected ileum. Lepromatous type. $\gamma\delta^+$ cells. (c) Paratuberculosis infected ileum. Tuberculoid type. $\gamma\delta^+$ cells.

Analysis of variance for the CD4 + CD8:$\gamma\delta$ ratios for the three groups of animals showed significant variance between these samples at $P < 0.05$ for total cells counted, and $P < 0.01$ for total villus cells. Two-sample analysis showed significant difference between control and lepromatous ratios for total cells ($P < 0.05$) and villus cells ($P < 0.01$), and between control and tuberculoid samples for villus cells only ($P < 0.05$). No significant differences were found between the lepromatous and tuberculoid groups.

3.5. Intraepithelial lymphocytes

No statistically significant differences were found in the relative percentages of the intraepithelial cell population between normal gut and paratuberculosis infected gut. The intraepithelial population was a relatively constant one, the mean percentages of all animals were: $CD4^+$ negligible (< 1%), $CD8^+$ 65.8%, and $\gamma\delta^+$ 33.2%.

4. Discussion

Paratuberculosis is a spectral disease with a range of lesions broadly comparable with that described for leprosy (Ridley and Jopling, 1966). As in leprosy, two polar forms exist, tuberculoid and lepromatous, and are manifest as distinct histological entities, suggesting that different immune mechanisms are involved in the pathogenesis of each form.

Tuberculoid type lesions in the intestine had increased densities of all T-cell subsets but were characterised by a cellular infiltrate that is predominantly $CD4^+$ in nature, with a decrease in the relative percentage of $CD8^+$ cells. There was a marked cellular infiltrate and often very few acid-fast bacilli detectable by Ziehl-Neelsen staining. There is some evidence in support of the functional subdivision of ruminant $CD4^+$ helper cells into Th1 and Th2 subsets (Brown et al., 1993; Brown et al., 1994). The lesions seen in the tuberculoid form may be due to $CD4^+$ cell activity similar to that described as a Th1 type response, resulting in efficient intracellular killing of the organism by infected macrophages but with associated delayed-type hypersensitivity and resultant immunopathology. $CD4^+$ cells have been shown to be the predominant infiltrating T-cell subset in human tuberculoid leprosy (Van Voorhis et al., 1982; Modlin et al., 1988) and this type of lesion is associated with high levels of IFN γ production (Salgame et al., 1991). In tuberculoid leprosy lesions mRNA for Th1 cytokines has been shown to predominate, whereas mRNA for Th2 cytokines was predominant in lepromatous lesions (Yamamura et al., 1991).

In lepromatous type lesions it was noted that, compared with non-infected control gut, there was a decrease in the $CD4^+$ and $CD8^+$ cell densities, which may be due to the presence of high numbers of large, infected macrophages acting as a space occupying lesion. The absolute numbers of the $\gamma\delta^+$ subset did not change significantly and there was no significant change in the relative percentages of $CD4^+$ and $CD8^+$ subsets compared with normal sheep, however there was a significant increase in the relative percentage of the $\gamma\delta^+$ subset. In lepromatous leprosy lesions the infiltrating cell type is predominantly $CD8^+$ (Van Voorhis et al., 1982; Modlin et al., 1988).

Lepromatous type pathology appears to correlate directly with higher levels of antibody production in ovine paratuberculosis (Clarke et al., 1996) suggesting that in lepromatous cases, the persistence of the pathogen may be explained by a misdirected, mainly Th2-like type of response, resulting in inappropriate antibody production. It has been shown that $CD8^+$ cells from lepromatous leprosy lesions can be activated to suppress $CD4^+$ T-cell proliferation in vitro (Modlin et al., 1986). On the basis of phenotype only, no significant difference in the CD4:CD8 ratios between lepromatous and non-infected animals was found, however this study was not an assessment of the functional activity of the lymphocyte subsets.

Gamma-delta T-cells are a prominent subset within the ruminant immune system (Hein and Mackay, 1991), have been shown to be involved in the protective response to mycobacterial infections (Janis et al., 1989; Modlin et al., 1989) and are known to respond to a range of mycobacterial antigens (Rust and Koning, 1993).

In a study of peripheral blood lymphocytes in bovine paratuberculosis, $\gamma\delta^+$ cells have been shown to exhibit a cytotoxic immunoregulatory function for $CD4^+$ cells (Chiodini and Davis, 1992). Likewise, the $\gamma\delta^+$ cell cytotoxicity was also subject to downregulation by $CD8^+$ cells (Chiodini and Davis, 1993). Increased numbers of $\gamma\delta^+$ T-cells in the intestine may be an indication that they play an active role in the local immune response to paratuberculosis.

The two histological forms of ovine paratuberculosis described were associated with different lamina propria lymphocyte populations. This suggests that distinct immune mechanisms are involved in the pathogenesis of each form. The effector functions of these populations require characterisation, and further investigations are in progress to determine the cytokine production in tuberculoid and lepromatous lesions of ovine paratuberculosis.

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